

AD_____

Award Number: W81XWH-04-1-0524

TITLE: The Role of BRCA1/BARD1 Heterodimers in the Mitosis-Interphase Transition

PRINCIPAL INVESTIGATOR: Vladimir Joukov, M.D., Ph.D.

CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
Boston, MA 02115

REPORT DATE: May 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				<i>Form Approved</i> OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01/05/07		2. REPORT TYPE Final		3. DATES COVERED (From - To) 23 Apr 2004 – 22 Apr 2007	
4. TITLE AND SUBTITLE The Role of BRCA1/BARD1 Heterodimers in the Mitosis-Interphase Transition				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-04-1-0524	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Vladimir Joukov, M.D., Ph.D. E-Mail: vladimir_joukov@dfci.harvard.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Dana-Farber Cancer Institute Boston, MA 02115				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT: The study was aimed at exploiting the advantages of the Xenopus egg extract as a biochemically tractable in vitro cell cycle model system in order to elucidate the molecular function of the breast and ovarian tumor suppressor BRCA1 and its heterodimerizing partner, BARD1. Experiments using both egg extracts and cultured mammalian cells revealed a previously unknown role of BRCA1/BARD1 in the mitotic spindle assembly. This BRCA1/BARD1 function is centrosome-independent, operates downstream of Ran GTPase, and depends upon the E3 ubiquitin ligase activity of the heterodimer. BRCA1/BARD1 ensures proper spindle pole formation by down-modulating the function of a recently discovered TPX2 partner, XRHAMM, thereby facilitating the accumulation of TPX2, the major spindle assembly factor and Ran target, on spindle poles. Our study implicated BRCA1/BARD1 in the regulation of three SAFs (RHAMM, TPX2, and Aurora A), which are overexpressed or amplified in certain cancers, thus pointing to the existence of a tumor suppressor-oncoprotein network that controls mitotic spindle assembly.					
15. SUBJECT TERMS BRCA1, BARD1, Tumor Suppressor, Genomic Instability, Mitosis, Mitotic spindle, TPX2, RHAMM, NuMA, Aurora A, Microtubule, Spindle Pole, RING Finger, Ubiquitin					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			USAMRMC
			UU	63	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	12
Reportable Outcomes.....	13
Conclusion.....	14
References.....	15
Appendices.....	18
Supporting Data.....	53

INTRODUCTION

Germ line mutations in the BRCA1 gene predispose to breast and/or ovarian cancer (Miki, et al., 1994). The most commonly accepted view attributes the tumor suppressor function of BRCA1 to its role in the maintenance of genomic integrity via participation in homologous recombination-mediated double strand break repair, in cell cycle checkpoint responses, and in the regulation of centrosome duplication (Reviewed in (Deng and Wang, 2003; Venkitaraman, 2002)). Mouse knock-out experiments revealed that BRCA1 is essential for cell viability and proliferation, an observation that seems at odds with the tumor suppressor role of BRCA1 ((Deng and Wang, 2003; Joukov, et al., 2001; Ludwig, et al., 1997; McCarthy, et al., 2003; Venkitaraman, 2002) and references therein). *In vivo*, most BRCA1 molecules form complexes with a structurally related protein, BARD1, and the resulting heterodimers exhibit E3 ubiquitin ligase activity of unknown physiological substrate specificity (Hashizume, et al., 2001; Mallery, et al., 2002; Sato, et al., 2004; Starita, et al., 2004; Yu, et al., 2006). Despite extensive studies, it is still unclear how BRCA1/BARD1 operates at the molecular level. These studies, however, have been hindered by the non-viability of the mammalian cells and embryos that lack the heterodimer. In order to overcome this problem, we set out to adapt the *Xenopus* egg extract as a biochemically tractable cell-free system for dissecting the molecular function of BRCA1/BARD1. This study has uncovered a novel function for BRCA1/BARD1 in mitosis that likely contributes to its role in chromosomal stability control and tumor suppression.

BODY

The study was aimed at understanding the molecular function of the BRCA1/BARD1 heterodimer at the mitosis-to-interphase transition. It was carried out in accordance with the specific aims and the tasks outlined in the Proposal and the Statement of Work. Our initial hypothesis that BRCA1/BARD1 controls certain chromatin-associated proteins such as RCC1, ORC, and HP1 was based on the observation that loading of these

proteins onto postmitotic chromatin occurred in a BRCA1/BARD1-dependent manner. However, upon scrutinizing this hypothesis, we obtained several lines of evidence that this dependence is indirect as BRCA1/BARD1 is essential for proper nuclear assembly in postmitotic interphase, and the efficient loading of RCC1, ORC, and HP1 (as well as other proteins) onto chromatin, in turn, requires proper formation of the nucleus. Indeed, when naïve egg extract was treated with agents that disrupt nuclear assembly (e.g. wheat germ agglutinin), the loading of RCC1, ORC2, and HP1 onto postmitotic chromatin was decreased to the degree similar to that seen in BRCA1/BARD1-depleted extract.

Our subsequent studies revealed that BRCA1/BARD1 has a major role in mitosis, when it functions outside chromatin by regulating spindle assembly factors XRHAMM and TPX2, thereby facilitating accumulation of TPX2 on spindle poles and proper spindle pole formation. Consistent with the functional link between BRCA1/BARD1 and TPX2 and with the role of BRCA1/BARD1 in postmitotic nuclear assembly, a recent study has implicated TPX2 in postmitotic nuclear assembly (O'Brien and Wiese, 2006), thus suggesting that the postmitotic defects seen in BRCA1/BARD1-deficient extract could be due to dysregulation of TPX2.

The main research accomplishments of the study are summarized in the recently published article (Joukov, et al., 2006). Below is the summarized report on each task outlined in the Statement of work.

Task 1. To find components and conditions that permit rescue of the postmitotic defects caused by BRCA1/BARD1 depletion

1.1. Rescue of the defects with recombinant wt BRCA1/BARD1, and if feasible, clinically relevant mutants of these proteins

In order to examine whether the mitotic and postmitotic defects in BRCA1/BARD1-depleted extract were a specific result of eliminating BRCA1/BARD1, the result of removing of another protein, or due to non-specific effects resulting from the depletion procedure itself, we asked whether the aforementioned defects could be reversed with purified BRCA1/BARD1. To this end, we produced baculovirus-expressed, affinity purified *Xenopus* wt BRCA1/BARD1 heterodimer. As a control, we also generated a

heterodimer bearing a single amino acid substitution in the BRCA1 RING finger (I26A). BRCA1(I26A) heterodimerizes with BARD1 nearly as efficiently as the wt BRCA1, however, the resulting heterodimer exhibits significantly reduced E3 ubiquitin ligase activity as assessed by autoubiquitination and ubiquitination of histones H2A and H2B (See Figure S2 in the attached article (Joukov, et al., 2006)).

The postmitotic defects in the BRCA1/BARD1-depleted extract were significantly reversed by the wt BRCA1/BARD1 heterodimer (See Figures S1C and S1D in (Joukov, et al., 2006)). The wt heterodimer also efficiently restored the mitotic spindle assembly and TPX2 accumulation on spindle poles in BRCA1/BARD1 extract. In contrast, BRCA1(I26A)/BARD1 was significantly less efficient in these settings (See Figures 3A, 4, and 5B in (Joukov, et al., 2006)).

Taken together, these results demonstrate that i) the mitotic and postmitotic defects in immunodepleted extract are due to specific elimination of BRCA1/BARD1; and ii) the E3 activity of BRCA1/BARD1 is critical for its mitotic spindle-organizing function.

1.2 Rescue with purified Xenopus BRCA1/BARD1 complexes and

1.3. Characterization of BRCA1/BARD1 complexes

We purified the endogenous BRCA1/BARD1 complexes from *Xenopus* extracts using an affinity-purified antibody directed against N-terminal peptide sequence of *Xenopus* BRCA1 (EVTRLIPCRQKKPKKE). These complexes contained xBRCA2, consistent with the previously reported association between human BRCA1 and BRCA2 (Chen, et al., 1998). In addition, a variety of chromatin proteins such as histones H2A/H2B, HP1, and ORC2 were found in BRCA1/BARD1 complexes (Figure 1).

Since the recombinant baculoviral BRCA1/BARD1 was quite efficient in reversing defects occurring in immunodepleted extracts, we found it unnecessary to carry out similar rescue experiments with the endogenous BRCA1/BARD1 complexes.

In an alternative approach, we isolated BRCA1/BARD1 protein complexes from egg extract using the recombinant heterodimer as a bait. BRCA1(I26A)/BARD1 was used for this purpose as certain protein interactions with human BRCA1/BARD1 appeared to be stabilized by the I26A mutation (Sato, et al., 2004). The FLAG-tagged BRCA1(I26A)/BARD1 was bound to FLAG agarose, and the resulting affinity beads or

the control, “empty”, FLAG agarose beads were incubated with metaphase-arrested extract. The beads were retrieved, washed, and analyzed by staining and Western blotting. Some proteins bound to FLAG beads non-specifically, i.e. they bound to the heterodimer-loaded beads as efficiently as to the control beads (See Figure 2D in the 2005 annual report). Included were dynein, γ -tubulin, and TPX2. By contrast, other proteins, including NuMA and XRHAMM, exhibited preferential affinity towards BRCA1/BARD1 beads (Figure 2D in the 2005 annual report). These results supported our observation that the endogenous BRCA1/BARD1 forms complexes with the spindle assembly factors NuMa and XRHAMM *in vivo* (See Figure 6 in (Joukov, et al., 2006)).

Task 2. To analyze molecular link between BRCA1/BARD1 and the previously identified proteins that might be involved in BRCA1/BARD1- specific postmitotic defects

2.1 Production of recombinant bacterial Ran and RCC1 proteins

We have produced bacterially-expressed 6xHis+S-tagged Ran and RCC1 proteins, as well as the corresponding rabbit polyclonal antibodies and used these reagents for probing the functional link between BRCA1/BARD1 and the Ran pathway (Figure 2 and (Joukov, et al., 2006)). As expected, the recombinant RCC1 bound Ran and a set of Ran-binding proteins including RanBP2 and importin β (Figure 2).

2.2 Probing the link with the Ran pathway

We observed that BRCA1/BARD1-depleted extracts and cells share major phenotypic features with cells in which the Ran pathway or certain downstream targets of Ran-GTP were disrupted. These features included mitotic spindle defects, chromosome missegregation, and micronucleus formation (Compton and Cleveland, 1993; Merdes and Cleveland, 1998; Moore, et al., 2002; O'Brien and Wiese, 2006; Wang, et al., 2004). Consistent with these observations, binding of RCC1 to mitotic chromatin was compromised in BRCA1/BARD1-deficient settings (See the 2004 Annual report). Importantly, the postmitotic defects in BRCA1/BARD1-depleted extract were rescued by supplementation of the extract with recombinant RanGTP (See the 2004 Annual report, Figure 4).

We initially suspected that the mitotic and postmitotic defects seen in BRCA1/BARD1 depleted extract were due to the deficient loading of RCC1 onto chromatin, (i.e. occurred *upstream* of RanGTP). Our subsequent studies however revealed that BRCA1/BARD1 controls the Ran-dependent spindle assembly *downstream* of RanGTP. This conclusion was based on the observation that microtubule structures induced by the addition of a constitutively active (defective in GTP hydrolysis) Ran mutant (Ran(Q69L)-GTP) to the BRCA1/BARD1-depleted extract, were highly disorganized and lacked defined poles (See Figure 4 in (Joukov, et al., 2006)).

How BRCA1/BARD1 controls binding of RCC1 to chromatin and whether this activity relates to the microtubule-organizing function of the heterodimer, is currently unknown. In the light of the recently demonstrated spike of RCC1 loading onto mitotic chromatin, which follows chromosome alignment and releases the spindle assembly checkpoint (Arnaoutov and Dasso, 2003), one can speculate that the RCC1/chromatin loading defect is a consequence of chromosomal misalignment that occurs in the absence of BRCA1/BARD1. We will test this hypothesis in the future.

2.2 Testing the involvement of ORC and HP1

The effect of BRCA1/BARD1 depletion of the loading of ORC and HP1 onto postmitotic chromatin seems to be indirect (See below).

Task 3. Search for additional chromatin-associated BRCA1/BARD1 partners

3.1 Purification of the two previously detected chromatin species that undergo deubiquitination in the absence of BRCA1/BARD1 and

3.2 Optimizing conditions for the two-dimensional protein electrophoresis of ubiquitinated chromatin-associated proteins

Given that BRCA1/BARD1 binds chromatin in S phase, we initially searched for the substrates of its E3 ubiquitin ligase activity among other interphase chromatin-associated proteins. For this purpose, the advantage of *Xenopus* egg extracts as an

excellent experimental system for the analysis of protein/chromatin associations was exploited (Walter and Newport, 2000).

Sperm chromatin was incubated in the mock-treated or immunodepleted interphase extracts for 1 hr and isolated from the extracts by centrifugation through a sucrose cushion. Using two-dimensional gel electrophoresis, we were unable to detect any significant differences in the pattern of chromatin-associated proteins between two extracts (Figure 3). In an alternative approach, we searched for the effect of BRCA1/BARD1 depletion on ubiquitination of chromatin-bound proteins. The extracts were supplemented with ^{32}P -labeled ubiquitin prior to the addition of sperm chromatin. Chromatin was isolated as described above, and subjected to SDS-PAGE followed by autoradiography. Depleting BRCA1/BARD1 from interphase extracts did not affect ubiquitination of chromatin-associated proteins (Figure 4A).

In order to test whether BRCA1/BARD1 is required for the ubiquitination of chromatin-associated proteins in postmitotic interphase, we preincubated sperm chromatin in the metaphase-arrested mock-treated or BRCA1/BARD1-depleted extracts supplemented with ^{32}P -labeled ubiquitin for 1 hr, and released the arrest by the addition of Ca^{2+} . After additional 1 hr incubation, chromatin was isolated and analyzed as described above. In these settings, ubiquitination of three protein products was significantly compromised in BRCA1/BARD1-depleted extract (Figure 4B). Based on electrophoretic mobility of these proteins and their presence in the acid-soluble chromatin fraction, we suspected that the aforementioned proteins are ubiquitinated histone(s). This hypothesis was tested by two-dimensional gel electrophoretic of acid-soluble chromatin fractions, side-by-side with recombinant histones (Dimitrov and Wolffe, 1997), which were ubiquitinated *in vitro* by the recombinant BRCA1/BARD1 heterodimer (Mallery, et al., 2002). The ubiquitinated products that were affected by BRCA1/BARD1 depletion co-migrated with mono- and di-ubiquitinated histone H2B (Figure 5). The identity of the band #1 (Figure 4B) is currently unknown.

We found that in egg extract, histone H2B undergoes mono- and di-ubiquitination in interphase, and it is de-ubiquitinated in mitosis (Figure 6). The ubiquitination of histone H2B is efficient in low-speed interphase extract, but it is significantly reduced in high-speed interphase extract that lacks membranes, suggesting that this modification

depends upon nuclear assembly (data not shown). Indeed, treatment of naïve egg extract with wheat germ agglutinin, an inhibitor of nuclear transport and growth, prevented ubiquitination of histone H2B to the degree comparable to that observed in BRCA1/BARD1-depleted extract. These results strongly suggest that inefficient postmitotic ubiquitination of histone H2B is not a result of specific elimination of BRCA1/BARD1, but rather a consequence of the nuclear assembly defect that occurs in the absence of BRCA1/BARD1 function.

To our surprise, other consequences of BRCA1/BARD1 depletion such as deficient loading of RCC1, HP1, and failure of histone H3/Ser10 dephosphorylation in postmitotic interphase were also seen only in the *low-speed* extracts. In contrast, no differences in these parameters were detected between the *high-speed* mock-treated and BRCA1/BARD1-deficient extracts (Figure 7 and data not shown). Importantly, not only nuclear assembly, but also formation of the mitotic spindle is known to be compromised in the high-speed extract. Taken together, these observations led us to a suspicion that the postmitotic abnormalities in immunodepleted extract is a consequence of abnormal mitosis resulting from the loss of BRCA1/BARD1 function. Indeed, our subsequent observations revealed that BRCA1/BARD1 is critical for proper mitotic microtubule organization and spindle assembly, thus supporting the aforementioned notion (See the 2005 annual report and (Joukov, et al., 2006)).

3.3. Purification and identification of novel potential substrates of BRCA1/BARD1 E3 ubiquitin ligase

Our study revealed that BRCA1/BARD1 has a critical function in mitosis, which depends upon intact E3 ubiquitin ligase activity of the heterodimer (See the 2005 annual report and (Joukov, et al., 2006)). The requirement of BRCA1/BARD1 for proper spindle pole assembly and for TPX2 accumulation on spindle poles as well as its interaction with RHAMM, TPX2, and NuMA, strongly suggest that at least one of these proteins or another spindle assembly factor(s) may be a substrate(s) of the heterodimeric E3 ubiquitin-ligase activity.

A series of experiments involving a C-terminal fragment of XRHAMM and an antibody generated against this fragment, led us to a conclusion that

BRCA1/BARD1 attenuates the otherwise excessive activity of XRHAMM, thereby permitting normal accumulation of TPX2 on spindle poles and proper spindle pole formation (See the 2005 annual report; Figures 8, 9, and 10; and (Joukov, et al., 2006)). This function is centrosome-independent, operates downstream of Ran GTPase, and depends upon BRCA1/BARD1 E3 ubiquitin ligase activity (Joukov, et al., 2006).

Furthermore, our study implicated the highly conserved, leucine zipper-bearing, C-terminal domain of XRHAMM as a contributor to those aspects of microtubule (MT)-organizing function that are regulated by BRCA1/BARD1 (Figure 10 and (Joukov, et al., 2006)). Given that the leucine zipper is a potential protein-interacting motif, we speculate that the C-terminal domain of XRHAMM is involved in the formation of XRHAMM homodimers or heterodimers with other spindle assembly factors (SAFs) and that these interactions are important for targeting of TPX2 to spindle poles. In a similar vein, one way of explaining the XRHAMM-agonistic effect of XRHAMM-Cwt (Figure 10) is to propose that it interferes with the homo- and/or heterodimerization of endogenous XRHAMM.

Interestingly, three SAFs, which are regulated by BRCA1/BARD1 (RHAMM, TPX2, and Aurora A), are overexpressed or amplified in certain cancers (Reviewed in (Crane, et al., 2003); (Hall, et al., 1995; Maxwell, et al., 2005; Smith, et al., 2006)), suggesting the existence of a tumor suppressor-oncoprotein network that controls mitotic spindle assembly (Figure 11). The role of BRCA1/BARD1 in spindle formation and in the regulation of TPX2 accumulation on spindle poles was confirmed by experiments involving siRNA-mediated BRCA1/BARD1 depletion in HeLA cells (Figure 9B and (Joukov, et al., 2006)).

At this point, it is unclear how BRCA1/BARD1 regulates microtubule organization in the process of mitotic spindle assembly. Down-modulation of XRHAMM by BRCA1/BARD1 suggests a scenario whereby the heterodimer ubiquitinates either XRHAMM or its regulatory protein(s). Thus, BRCA1/BARD1 substrate is likely either one of the SAFs itself or a SAF-associated- or a MT-associated protein. Our future studies should shed light on the identity of the BRCA1/BARD1 mitotic substrate(s).

Task 4. Generation of recombinant proteins and production of specific antibodies

In the process of our study, we generated a variety of recombinant proteins and rabbit polyclonal antibodies directed against BRCA1, BARD1, and the suspected functional partners of BRCA1/BARD1 such as hRan, xRCC1, xRanBP2, xTPX2, XRHAMM, xNuMa, xKlp2, and xAurora A (See 2004 and 2005 annual reports and (Joukov, et al., 2006)).

KEY RESEARCH ACCOMPLISHMENTS

- The study provided the first demonstration of an essential mitotic function of the BRCA1/BARD1 heterodimer. BRCA1/BARD1 has been implicated in the control of microtubule- and spindle pole organization during mitotic spindle assembly.
- A mechanism underlying the spindle-organizing function of BRCA1/BARD1 has been uncovered. BRCA1/BARD1 down-modulates the activity of a TPX2 partner, XRHAMM, thereby facilitating accumulation of TPX2 on spindle poles and permitting proper spindle pole formation.
- The study implicated a tumor suppressor complex, BRCA1/BARD1, in the regulation of several spindle assembly factors (RHAMM, TPX2, and Aurora A), which have been previously linked to cancer in their own right, thus pointing to the existence of a tumor suppressor-oncoprotein network that controls mitotic spindle assembly.
- A reliable *in vitro* assay for the analysis of BRCA1/BARD1 mitotic function has been established.

REPORTABLE OUTCOMES

Scientific publications

The results of this study were published as an article in one of the top scientific journals:

- Joukov, V., Groen, A.C., Prokhorova, T., Gerson, R., White, E., Rodriguez, A., Walter, J.C., and Livingston, D.M. (2006) The BRCA1/BARD1 heterodimer modulates Ran-dependent mitotic spindle assembly. Cell 127, 539-552.

Conferences

The results of the study were presented at one national and at three international scientific meetings:

- LXX Cold Spring Harbor Symposium in Quantitative Biology - Molecular Approaches to Controlling Cancer (Cold Spring Harbor, New York, USA); June 1-6, 2005.
- The Era of Hope Department of Defense Breast Cancer Research Program Meeting (Philadelphia, Pennsylvania, USA); June 8-11, 2005.
- Cold Spring Harbor Laboratory Meeting - The Cell Cycle (Cold Spring Harbor, New York, USA); May 17-21, 2006.
- Cold Spring Harbor Laboratory Meeting - Mechanisms and Models of Cancer (Cold Spring Harbor, New York, USA); August 16-20, 2006 (Oral presentation).

Employment opportunities

The study funded by this grant allowed Dr. Joukov to be shortlisted for faculty positions in several top universities, where he is currently being interviewed. Dr. Joukov will continue this study in his newly established laboratory.

CONCLUSIONS

We exploited the *Xenopus* egg extract experimental system in order to understand how BRCA1/BARD1 controls cell proliferation and chromosomal stability. Analysis of extracts depleted of BRCA1/BARD1 using specific antibodies revealed that the heterodimer is essential for the fidelity of mitosis and mitotic exit, but it is dispensable for the interphase processes such as nuclear assembly and DNA replication. The study uncovered a previously unknown mitotic function of BRCA1/BARD1 in the regulation of microtubule and spindle pole organization. BRCA1/BARD1 attenuates the otherwise excessive activity of XRHAMM, thereby permitting normal accumulation of TPX2 on spindle poles and proper spindle pole formation. This function is centrosome-independent, operates downstream of Ran GTPase, and depends upon BRCA1/BARD1 E3 ubiquitin ligase activity. Moreover, the BRCA1/BARD1 MT-organizing activity can be detected even in the absence of chromatin and centrosomes, by using an assay, in which the assembly of spindle-like structures (MT asters) is driven by the hydrolysis-deficient Ran mutant, Ran(Q69L)GTP.

The role of BRCA1/BARD1 in spindle formation and in the regulation of TPX2 accumulation on spindle poles was confirmed by experiments involving siRNA-mediated BRCA1/BARD1 depletion in HeLA cells.

Our study implicated BRCA1/BARD1 in the regulation of three SAFs (RHAM, TPX2, and Aurora A), which are overexpressed or amplified in certain cancers, thus suggesting that the mitotic function of BRCA1/BARD1 might be an important contributor to its role as a tumor suppressor.

The results of this study may help in developing new approaches for cancer treatment. We are planning to use *Xenopus* egg extracts, particularly the MT aster formation/TPX2 localization assay, for phenotypic screens aimed at the identification of components that modulate BRCA1 mitotic targets such as RHAMM, TPX2, and Aurora A. Further work on the project will hopefully lead to the identification of mitotic substrates of the BRCA1/BARD1 E3 ubiquitin ligase and will shed new light on the molecular mechanisms involved in the maintenance of chromosomal stability and tumor suppression.

REFERENCES

- Arnaoutov, A., and Dasso, M. (2003). The Ran GTPase regulates kinetochore function. *Dev Cell* 5, 99-111.
- Chen, J., Silver, D. P., Walpita, D., Cantor, S. B., Gazdar, A. F., Tomlinson, G., Couch, F. J., Weber, B. L., Ashley, T., Livingston, D. M., and Scully, R. (1998). Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Molecular Cell* 2, 317-328.
- Compton, D. A., and Cleveland, D. W. (1993). NuMA is required for the proper completion of mitosis. *J Cell Biol* 120, 947-957.
- Crane, R., Gadea, B., Littlepage, L., Wu, H., and Ruderman, J. V. (2003). Aurora A, meiosis and mitosis. *Biol Cell* 96, 215-229.
- Deng, C. X., and Wang, R. H. (2003). Roles of BRCA1 in DNA damage repair: a link between development and cancer. *Hum Mol Genet* 12 *Spec No 1*, R113-123.
- Dimitrov, S. I., and Wolffe, A. P. (1997). Fine resolution of histones by two-dimensional polyacrylamide gel electrophoresis: developmental implications. *Methods* 12, 57-61.
- Hall, C. L., Yang, B., Yang, X., Zhang, S., Turley, M., Samuel, S., Lange, L. A., Wang, C., Curpen, G. D., Savani, R. C., *et al.* (1995). Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-ras transformation. *Cell* 82, 19-26.
- Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohta, T. (2001). The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem* 276, 14537-14540.
- Joukov, V., Chen, J., Fox, E. A., Green, J. B., and Livingston, D. M. (2001). Functional communication between endogenous BRCA1 and its partner, BARD1, during *Xenopus laevis* development. *Proc Natl Acad Sci USA* 98, 12078-12083.
- Joukov, V., Groen, A. C., Prokhorova, T., Gerson, R., White, E., Rodriguez, A., Walter, J. C., and Livingston, D. M. (2006). The BRCA1/BARD1 heterodimer modulates ran-dependent mitotic spindle assembly. *Cell* 127, 539-552.
- Ludwig, T., Chapman, D. L., Papaioannou, V. E., and Efstratiadis, A. (1997). Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of *Brca1*, *Brca2*, *Brca1/Brca2*, *Brca1/p53*, and *Brca2/p53* nullizygous embryos. *Genes Dev* 11, 1226-1241.

Mallery, D. L., Vandenberg, C. J., and Hiom, K. (2002). Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *EMBO J* 21, 6755-6762.

Maxwell, C. A., Keats, J. J., Belch, A. R., Pilarski, L. M., and Reiman, T. (2005). Receptor for hyaluronan-mediated motility correlates with centrosome abnormalities in multiple myeloma and maintains mitotic integrity. *Cancer Res* 65, 850-860.

McCarthy, E. E., Celebi, J. T., Baer, R., and Ludwig, T. (2003). Loss of Bard1, the heterodimeric partner of the Brca1 tumor suppressor, results in early embryonic lethality and chromosomal instability. *Mol Cell Biol* 23, 5056-5063.

Merdes, A., and Cleveland, D. W. (1998). The role of NuMA in the interphase nucleus. *J Cell Sci* 111 (Pt 1), 71-79.

Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P. A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L. M., and Ding, W. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* 266, 66-71.

Moore, W., Zhang, C., and Clarke, P. R. (2002). Targeting of RCC1 to chromosomes is required for proper mitotic spindle assembly in human cells. *Curr Biol* 12, 1442-1447.

O'Brien, L. L., and Wiese, C. (2006). TPX2 is required for postmitotic nuclear assembly in cell-free *Xenopus laevis* egg extracts. *J Cell Biol* 173, 685-694.

Sato, K., Hayami, R., Wu, W., Nishikawa, T., Nishikawa, H., Okuda, Y., Ogata, H., Fukuda, M., and Ohta, T. (2004). Nucleophosmin/B23 is a candidate substrate for the BRCA1-BARD1 ubiquitin ligase. *J Biol Chem* 279, 30919-30922.

Smith, L. T., Mayerson, J., Nowak, N. J., Suster, D., Mohammed, N., Long, S., Auer, H., Jones, S., McKeegan, C., Young, G., *et al.* (2006). 20q11.1 amplification in giant-cell tumor of bone: Array CGH, FISH, and association with outcome. *Genes Chromosomes Cancer* 45, 957-966.

Starita, L. M., Machida, Y., Sankaran, S., Elias, J. E., Griffin, K., Schlegel, B. P., Gygi, S. P., and Parvin, J. D. (2004). BRCA1-dependent ubiquitination of gamma-tubulin regulates centrosome number. *Mol Cell Biol* 24, 8457-8466.

Venkitaraman, A. R. (2002). Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 108, 171-182.

Walter, J., and Newport, J. (2000). Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Mol Cell* 5, 617-627.

Wang, R. H., Yu, H., and Deng, C. X. (2004). A requirement for breast-cancer-associated gene 1 (BRCA1) in the spindle checkpoint. *Proc Natl Acad Sci USA* *101*, 17108-17113.

Yu, X., Fu, S., Lai, M., Baer, R., and Chen, J. (2006). BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtlP. *Genes Dev* *20*, 1721-1726.

The BRCA1/BARD1 Heterodimer Modulates Ran-Dependent Mitotic Spindle Assembly

Vladimir Joukov,¹ Aaron C. Groen,² Tatyana Prokhorova,^{3,4} Ruth Gerson,¹ Erinn White,¹ Alison Rodriguez,¹ Johannes C. Walter,^{3,*} and David M. Livingston^{1,*}

¹Dana-Farber Cancer Institute and Harvard Medical School, 44 Binney Street, Boston, MA 02115, USA

²Department of Systems Biology

³Department of Biological Chemistry and Molecular Pharmacology

Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA

⁴Present address: The Molecular Endocrinology Unit, Medical Biotechnology Centre, University of Southern Denmark, 5000 Odense, Denmark.

*Contact: johannes_walter@hms.harvard.edu (J.C.W.), david_livingston@dfci.harvard.edu (D.M.L.)

DOI 10.1016/j.cell.2006.08.053

SUMMARY

The heterodimeric tumor-suppressor complex BRCA1/BARD1 exhibits E3 ubiquitin ligase activity and participates in cell proliferation and chromosome stability control by incompletely defined mechanisms. Here we show that, in both mammalian cells and *Xenopus* egg extracts, BRCA1/BARD1 is required for mitotic spindle-pole assembly and for accumulation of TPX2, a major spindle organizer and Ran target, on spindle poles. This function is centrosome independent, operates downstream of Ran GTPase, and depends upon BRCA1/BARD1 E3 ubiquitin ligase activity. *Xenopus* BRCA1/BARD1 forms endogenous complexes with three spindle-pole proteins, TPX2, NuMA, and XRHAMM—a known TPX2 partner—and specifically attenuates XRHAMM function. These observations reveal a previously unrecognized function of BRCA1/BARD1 in mitotic spindle assembly that likely contributes to its role in chromosome stability control and tumor suppression.

INTRODUCTION

Loss of BRCA1 function predisposes to breast and/or ovarian cancer (Miki et al., 1994). How BRCA1 exerts its tumor-suppression function remains incompletely understood. The most commonly accepted view attributes this function to a BRCA1 role in the maintenance of genomic integrity via participation in homologous-recombination-mediated double-strand-break repair, the regulation of cell-cycle checkpoint responses, and centrosome amplification control (reviewed in Deng and Wang, 2003; Venkitaraman, 2002).

In vivo, most BRCA1 molecules form heterodimers with a structurally related protein, BARD1 (Wu et al., 1996).

BRCA1 and BARD1 each contain an N-terminal RING domain and two C-terminal BRCT motifs. RING domains catalyze ubiquitin transfer by interacting with ubiquitin-conjugating enzymes, and BRCT domains can bind certain phosphoserine/phosphothreonine-containing peptide sequences (reviewed in Fang et al., 2003; Glover et al., 2004). BRCA1/BARD1 heterodimers promote ubiquitin transfer far more efficiently than either protein alone and can catalyze autoubiquitination as well as the cell-free ubiquitination of other proteins (Hashizume et al., 2001; Mallery et al., 2002; Sato et al., 2004; Starita et al., 2004; Yu et al., 2006). Whether any of these proteins is a physiological BRCA1/BARD1 substrate is unknown.

BRCA1 and BARD1 are conserved in vertebrates, plants, and worms but are absent from yeast (Boulton et al., 2004; Joukov et al., 2001). Inactivation of BRCA1 and BARD1 in mice and frogs yields similar phenotypes, with embryos dying early in embryogenesis. These embryos also reveal marked chromosomal abnormalities and a cell proliferation defect (Deng and Wang, 2003; Joukov et al., 2001; Ludwig et al., 1997; McCarthy et al., 2003; Venkitaraman, 2002 and references therein). The mechanism underlying these abnormalities is incompletely defined, although accumulating DNA damage that in turn activates cell-cycle checkpoints has been suggested (reviewed in Deng and Wang, 2003; Venkitaraman, 2002).

Although it was initially believed that BRCA1 functions largely in S phase (Scully et al., 1997; Venkitaraman, 2002), growing evidence suggests that it is also active in mitosis. First, aneuploidy is common among BRCA1- and BARD1-deficient cells (Joukov et al., 2001; McCarthy et al., 2003; Xu et al., 1999). Second, mouse fibroblasts that carry a biallelic hypomorphic *BRCA1* mutation exhibit mitotic defects (Xu et al., 1999). Third, BRCA1 binds to tubulin and localizes in part at centrosomes and spindle microtubules (Hsu and White, 1998). Fourth, steady-state levels of BRCA1 remain elevated through mitosis, whereas the protein is ubiquitinated and undergoes proteasome-dependent degradation in G1 and S phase (Choudhury

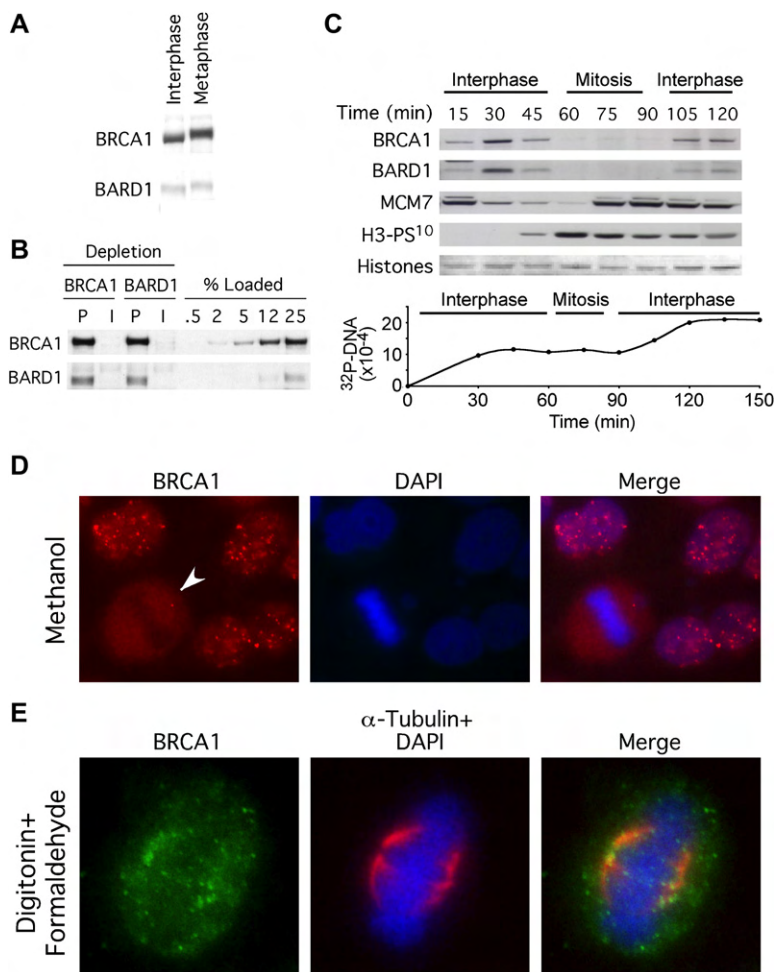


Figure 1. Cell-Cycle-Dependent Properties of BRCA1/BARD1

(A) Western blot (W blot) analysis of BRCA1 and BARD1 in interphase- and metaphase-arrested egg extract.

(B) BRCA1 and BARD1 associate with each other in egg extract. Interphase egg extract was depleted with XBRCA1- or XBARD1-specific Ab (I) or the corresponding preimmune IgG (P). One microliter of each sample and the indicated amount of untreated extract (% of 1 μ l) were analyzed by W blotting.

(C) Cell-cycle-dependent regulation of BRCA1/BARD1 chromatin binding. Chromatin isolated from cycling egg extract at the indicated time points was analyzed by W blotting with the indicated antibodies (upper panel). Aliquots of extract were also analyzed for DNA replication and chromatin morphology (lower panel and data not shown).

(D and E) Immunofluorescence microscopic images showing localization of BRCA1 in mitotic and S phase HeLa cells. Cells were fixed in methanol/acetone (D) or paraformaldehyde following permeabilization with digitonin (E) and stained with the indicated antibodies and DAPI.

et al., 2004). Finally, in contrast to normal proliferating somatic cells that are not viable without BRCA1 or BARD1, trophoblast giant cells, which endoreduplicate their DNA without intervening mitoses, remain unaffected when depleted of either protein (Ludwig et al., 1997; McCarthy et al., 2003).

We have examined the function of BRCA1/BARD1 heterodimers using *Xenopus* egg extracts as an experimental system (Murray, 1991). This study demonstrates that BRCA1/BARD1 ensures fidelity of mitosis and mitotic exit by regulating Ran-dependent (chromatin-driven) spindle assembly. BRCA1/BARD1 attenuates the activity of XRHAMM (*Xenopus* receptor for hyaluronic-acid-mediated motility) (Groen et al., 2004; Maxwell et al., 2003), thereby permitting the normal concentration of TPX2 (Wittmann et al., 2000) on spindle poles and proper spindle-pole assembly.

RESULTS

Cell-Cycle-Dependent Regulation of BRCA1/BARD1

Xenopus egg extracts were used to assess BRCA1/BARD1 function because this system faithfully reca-

pitulates cellular processes in which BRCA1/BARD1 is potentially involved. Importantly, these extracts allow one to bypass the problem of nonviability of BRCA1- and BARD1-deficient cells and embryos, which normally complicates in vivo studies of these proteins. The levels of BRCA1 and BARD1 were similar in extracts arrested in interphase and meiotic metaphase (Figure 1A). A BRCA1 antibody (Ab) depleted ~98% of the ambient BRCA1 and BARD1 in both interphase- and metaphase-arrested extracts, and a BARD1 Ab led to similar effects (Figure 1B and data not shown). These results imply that nearly all of the BRCA1 and BARD1 in *Xenopus* egg extracts exists in a heterodimeric complex throughout the cell cycle. In cycling egg extract that oscillates between S phase and mitosis due to the periodic synthesis and degradation of cyclin B (Murray, 1991), BRCA1 and BARD1 efficiently bound to chromatin in interphase and largely dissociated from it in mitosis (Figure 1C).

In cultured mammalian cells, BRCA1 formed characteristic foci in a subset of interphase cells as reported (Scully et al., 1997) (Figure 1D) and was diffusely distributed throughout the cell and excluded from chromatin during mitosis (Figure 1D, arrowhead). When soluble proteins

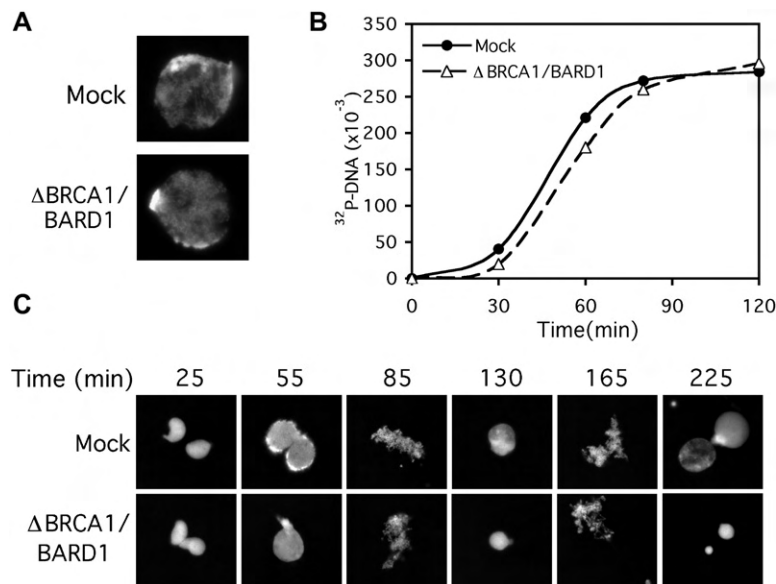


Figure 2. BRCA1/BARD1 Is Required for Postmitotic Nuclear Assembly

(A and B) BRCA1/BARD1 is dispensable for nuclear assembly and DNA replication in interphase egg extract. Aliquots of mock-treated and BRCA1/BARD1-depleted interphase extracts were withdrawn 90 min (A) or at the indicated times (B) after addition of sperm chromatin and analyzed for nuclear morphology (A) and DNA replication (B).

(C) Cycling extracts were supplemented with sperm chromatin. Aliquots were removed at the indicated times and analyzed for chromatin morphology.

were eluted from cells by digitonin permeabilization prior to fixation, residual BRCA1 was detected in metaphase cells in foci distributed around, but not on, chromatin or the mitotic spindle (Figure 1E).

The electrophoretic mobility of BRCA1 and BARD1 decreased as a result of specific phosphorylation during mitosis (Figure 1A and data not shown), when the heterodimer is largely excluded from chromatin (Figure 1C). Thus, in egg extract, the heterodimer accumulates in the nucleus and binds to interphase chromatin. In mitosis, most BRCA1/BARD1 is phosphorylated and excluded from chromatin. In mammalian cells, some mitotic BRCA1/BARD1 is localized in foci that surround chromatin and the spindle.

BRCA1/BARD1 Is Required for Proper Nuclear Assembly in Postmitotic Interphase

BRCA1- and BARD1-specific antibodies (Joukov et al., 2001) were used to deplete the heterodimer from extract (Figure 1B), and chromatin dynamics during interphase and mitosis was analyzed. When demembranated sperm chromatin was added to BRCA1/BARD1-depleted interphase extract, chromatin decondensation, nuclear-envelope formation, and the rate of DNA replication were the same as in mock-treated extract (Figures 2A and 2B). Thus, BRCA1/BARD1 is dispensable for S phase progression in egg extract. Similarly, when sperm chromatin was added to mock-treated or BRCA1/BARD1-depleted cycling extract, it efficiently decondensed and formed nuclei (Figure 2C, 25 min and 55 min). Both extracts subsequently entered mitosis as seen by nuclear-envelope breakdown and chromatin condensation (Figure 2C, 85 min). The extracts exited mitosis and continued to cycle nearly synchronously. Upon mitotic exit, nuclei of relatively uniform size formed in the mock-treated extract (Figure 2C, upper row, 130 min and 225 min). In contrast,

postmitotic nuclei in BRCA1/BARD1-depleted extract were heterogeneous, with the majority being 3 to 10 times smaller than control nuclei or nuclei formed in the immunodepleted extract during the first, premitotic interphase (Figure 2C, lower row, 130 min and 225 min versus 55 min).

Postmitotic interphase can be also generated by preincubating sperm chromatin in metaphase-arrested (also referred to as cytotstatic factor [CSF]-arrested) egg extract followed by release into interphase (detailed in the [Supplemental Experimental Procedures](#) in the [Supplemental Data](#) available with this article online). In such settings, uniform nuclei were observed in mock-treated extract. In contrast, nuclei that formed in BRCA1/BARD1-depleted extract upon release from the metaphase arrest varied in size, being 3 to 10 times smaller compared to the control nuclei (Figure S1A). Importantly, this defect was reversed by supplementing the depleted extract with immunoaffinity purified, recombinant *Xenopus* BRCA1/BARD1 heterodimer (rBRCA1/BARD1, detailed below) (Figures S1C and S1D; see also [Supplemental Results](#)).

Taken together, these results demonstrate that, in *Xenopus* egg extract, BRCA1/BARD1 is dispensable for certain interphase functions such as nuclear assembly and DNA replication. However, passage of chromatin through mitosis establishes a requirement for BRCA1/BARD1 for proper nuclear assembly.

BRCA1/BARD1 Is Required for Proper Mitotic Spindle Assembly

Given BRCA1/BARD1's involvement in postmitotic interphase, it was important to determine whether the heterodimer is also required for the execution of mitosis itself. To this end, we examined the effect of BRCA1/BARD1 depletion on metaphase spindle assembly. A standard approach that includes replication of sperm-chromatin DNA in interphase extract followed by the addition of

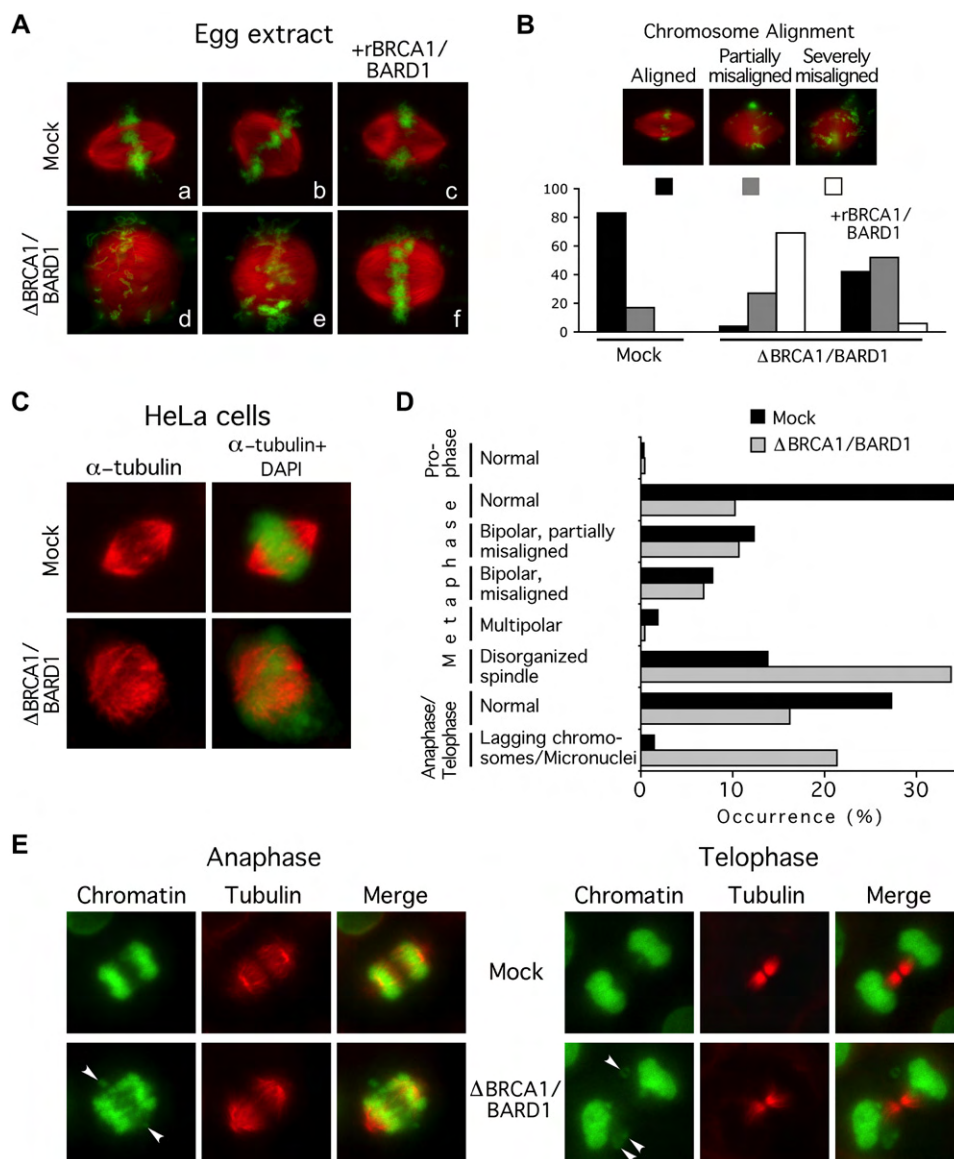


Figure 3. BRCA1/BARD1 Is Required for Proper Mitotic Spindle Assembly

(A and B) Sperm chromatin was replicated in mock-treated and BRCA1/BARD1-depleted, CSF-arrested extracts supplemented with rhodamine-labeled tubulin and, where indicated, rBRCA1/BARD1. The extracts were driven into metaphase and were analyzed 1 hr later for spindle morphology.

(A) Representative metaphase spindles.

(B) Spindle structures were categorized based on the degree of chromosome alignment (top panel) and were quantified (bottom panel).

(C–E) Mitotic defects in BRCA1/BARD1 siRNA-transfected HeLa cells.

(C) Representative images of normal (top panels) and disorganized (bottom panels) metaphase spindles assembled in mock-treated and BRCA1/BARD1-depleted cells, respectively.

(D) Quantitative analysis of normal and abnormal mitotic spindle structures that appeared in mock-treated versus BRCA1/BARD1-depleted cells. Examples are shown in Figure S3B.

(E) Representative examples of chromosome segregation defects in BRCA1/BARD1-deficient cells (arrowheads). Note inefficient focusing of microtubules into spindle poles in anaphase (Δ BRCA1/BARD1, “Tubulin” panel). In all images, microtubules are in red and chromatin is pseudocolored green.

CSF-arrested extract to induce the metaphase state was employed (Desai et al., 1999). In the mock-treated extract, most spindles were largely bipolar with focused spindle poles, and chromosomes properly congressed at the metaphase plate (Figures 3Aa and 3Ab). In contrast, most

spindles in the BRCA1/BARD1-depleted extract contained unfocused spindle poles and were more rounded. They also exhibited a higher density of microtubules and a failure of chromosome congression at the metaphase plate (Figures 3Ad and 3Ae). To confirm that the spindle

defect observed in immunodepleted extract was a specific outcome of eliminating BRCA1/BARD1, a recombinant heterodimer was produced by coexpression of *Xenopus* BRCA1 and BARD1 in cultured insect cells followed by immunoaffinity purification. *Xenopus* rBRCA1/BARD1 (Figure S2A, lane 2), like its human counterpart (Mallery et al., 2002), both displayed autoubiquitination and ubiquitinated certain histones, implying that it is biologically active (Figure S2B, lanes 2 and 4). The mitotic spindle phenotype was substantially alleviated by supplementing depleted extract with rBRCA1/BARD1 (Figure 3A). The effect of BRCA1/BARD1 depletion and rescue on chromosome alignment is quantified in Figure 3B.

We asked whether the mitotic spindle defects seen in depleted *Xenopus* egg extracts could be observed in BRCA1/BARD1-deficient cells. HeLa cells were transfected with control or BRCA1- and BARD1-specific siRNA oligonucleotides (Figure S3A), and mitotic spindle morphology was assessed. The mitotic figures were categorized based on the stage of mitosis in which they were detected (i.e., prophase, metaphase, anaphase, and telophase) and the extent to which they were defective (Figure S3B). The percentage of mitotic figures in each category was calculated (Figure 3D). BRCA1/BARD1 depletion did not affect the morphology or proportion of cells in prophase. However, it reduced from 35% to 10% the abundance of normal metaphase cells with bipolar spindles and properly aligned chromosomes. Accordingly, BRCA1/BARD1-deficient cells displayed a higher proportion of disorganized mitotic spindles (34% versus 14%). The metaphase spindle defects in HeLa cells and *Xenopus* egg extracts were remarkably similar (Figure 3C versus Figure 3A). In addition, BRCA1/BARD1 siRNA-treated cells exhibited a severe defect in chromosome segregation during anaphase, revealing chromosomal bridges and lagging chromosomes (Figures 3D and 3E, arrowheads in the “Anaphase” panel). At telophase, some lagging chromosomes became enclosed in nuclear envelopes, giving rise to micronuclei (Figure 3E, arrowheads in “Telophase” panel).

Taken together, these results indicate that BRCA1/BARD1 is required during mitosis for proper mitotic spindle assembly and at the mitosis-to-interphase transition for proper chromosome segregation and nuclear assembly.

BRCA1/BARD1 Regulates Ran-Driven Microtubule Organization

We observed that BRCA1/BARD1-depleted extracts and cells share major phenotypic features with cells in which the Ran pathway or certain downstream targets of Ran-GTP are disrupted. These features include mitotic spindle defects, chromosome missegregation, and micronucleus formation (Compton and Cleveland, 1993; Merdes and Cleveland, 1998; Moore et al., 2002; O'Brien and Wiese, 2006; Wang et al., 2004). We therefore asked whether BRCA1/BARD1 is involved in Ran-dependent spindle assembly. Addition of Ran-GTP to *Xenopus* egg extract is sufficient to cause the formation of spindle-related struc-

tures, called asters and pseudospindles, in the absence of DNA and centrosomes (Carazo-Salas et al., 1999; Wilde and Zheng, 1999). This phenomenon imitates chromatin-driven spindle assembly and is likely dependent upon achieving high local concentrations of spindle assembly factors (SAFs) (e.g., NuMA and TPX2) following their release by Ran-GTP from inhibitory binding by the importin α/β heterodimer (reviewed in Fant et al. 2004; Hetzer et al., 2002; Quimby and Dasso, 2003). When a constitutively active Ran mutant defective in GTP hydrolysis (Ran(Q69L)-GTP) was added to mock-treated extract, asters with radially oriented microtubules and sharply focused poles formed (Figure 4Aa), as reported (Carazo-Salas et al., 1999). In contrast, asters assembled in BRCA1/BARD1-depleted extract appeared larger in size and contained dense, disoriented microtubules with poorly focused poles (Figures 4Ab, 4Ac, 4B, and 4C). In the most severe cases, asters lacked defined centers (Figures 4Ac and 4Bd). Importantly, the number of Ran-induced asters was not affected by BRCA1/BARD1 depletion (data not shown), implying that BRCA1/BARD1 is not essential for microtubule (MT) assembly per se. Addition of wild-type (WT) rBRCA1/BARD1 to depleted extract significantly restored aster MT organization in terms of both qualitative appearance (Figure 4B) and absolute size (Figure 4C). Furthermore, a mutant rBRCA1(I26A)/BARD1 heterodimer selectively defective in ubiquitin transfer (Brzovic et al., 2003; Figure S2A, lane 3; Figure S2B, lanes 3 and 5 versus lanes 2 and 4) was significantly less efficient than its WT counterpart in rescuing these defects (Figures 4B–4D). These results indicate that BRCA1/BARD1 and its E3 ubiquitin ligase activity participate in MT organization and spindle-pole assembly downstream of Ran-GTP.

BRCA1/BARD1 Controls Targeting of TPX2 to Spindle Poles

Whether proteins involved in spindle-pole organization are affected by BRCA1/BARD1 depletion was investigated next. NuMA and TPX2 both participate in spindle-pole assembly and are targets of Ran during mitosis (Fant et al., 2004; Hetzer et al., 2002; Merdes et al., 2000; Wittmann et al., 2000). In addition, XRHAMM was recently implicated in chromatin-driven MT nucleation and spindle-pole formation (Groen et al., 2004). During mitosis, when the nuclear envelope disassembles, XRHAMM binds to microtubules and, in association with γ -TuRC and TPX2, facilitates Ran-dependent MT nucleation and concentration of TPX2 on spindle poles via a currently unknown mechanism (Groen et al., 2004; Maxwell et al., 2003). In mock-treated extract, NuMA, γ -tubulin, XRHAMM, and TPX2 efficiently bound to microtubules and concentrated on aster poles (Figures 5A and 5B). In BRCA1/BARD1-depleted extract, these proteins also bound to microtubules: NuMA accumulated on the aster poles almost as efficiently as in mock-treated extracts (Figure 5A, row 2 versus 1); XRHAMM and γ -tubulin were also concentrated on aster poles, although in a more diffuse and less orderly manner compared to mock-treated extract (Figure 5A,

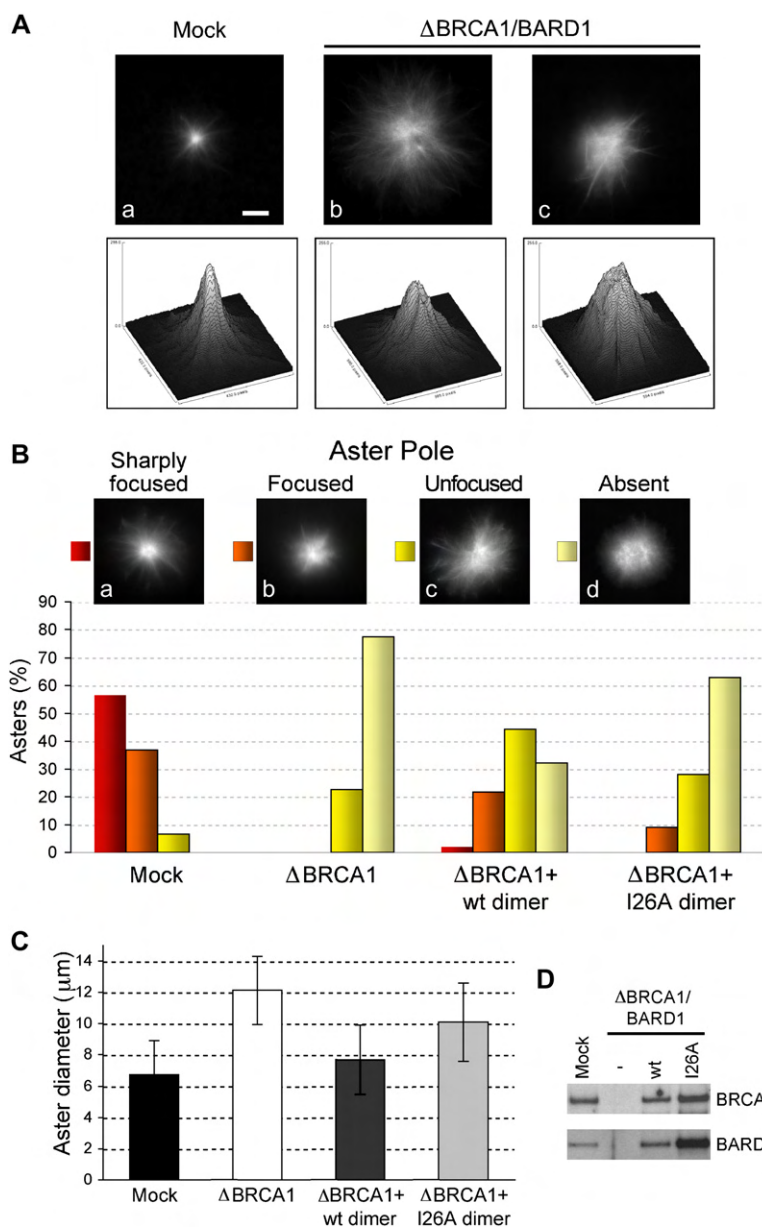


Figure 4. BRCA1/BARD1 Regulates Ran-Dependent MT Organization

(A) Fluorescence micrographs (top panels) and 3D surface plots (bottom panels) of the representative Ran-GTP-induced MT asters assembled in mock-treated and BRCA1/BARD1-depleted extracts. Scale bar = 10 μ m.

(B–D) Rescue of the MT aster structures with recombinant BRCA1/BARD1.

(B) Ran-GTP-induced MT asters assembled in mock-treated and BRCA1/BARD1-depleted extract were supplemented with buffer or with recombinant WT or enzymatically deficient (I26A dimer) BRCA1/BARD1. Asters were categorized based on the degree of aster-pole focusing (upper panel), and each category was quantified (lower panel).

(C) Average diameter of asters in each group. Error bars represent standard deviations ($n = 120$).

(D) W blot analysis of the extracts.

row 4 versus 3). In contrast, TPX2 was diffusely localized along the length of microtubules and failed to concentrate on aster poles (Figure 5B, row 2 versus 1). Addition of WT rBRCA1/BARD1 restored both aster MT organization and the concentration of TPX2 on aster poles, whereas rBRCA1(I26A)/BARD1 was less efficient compared to the WT heterodimer in rescuing both defects (Figure 5B, row 3 versus 2 and 4).

As stated above, Ran-GTP-induced aster formation utilizes the chromatin-driven/anastral pathway of spindle assembly. This pathway operates in cells that lack a defined MT-organizing center (MTOC), like oocytes of insects and vertebrates and cells of higher plants. In contrast, in most somatic cells, which contain centrosomes, the anas-

tral pathway likely cooperates with the MTOC-driven pathway of spindle assembly, with the latter being predominant (reviewed in Fant et al., 2004). We therefore asked whether, in the presence of centrosomes, BRCA1/BARD1 is also needed for efficient TPX2 accumulation on spindle poles. Notably, each sperm pronucleus contains a centrosome attached to its surface. Thus, MT architecture and TPX2 localization were compared in asters induced by sperm chromatin in mock-treated and BRCA1/BARD1-depleted CSF-arrested extracts. Although both extracts supported formation of microtubular structures around chromatin with similar efficiency, there was a profound difference in their architecture. Asters and spindles in the mock-treated extract had radial microtubules and sharply focused poles,

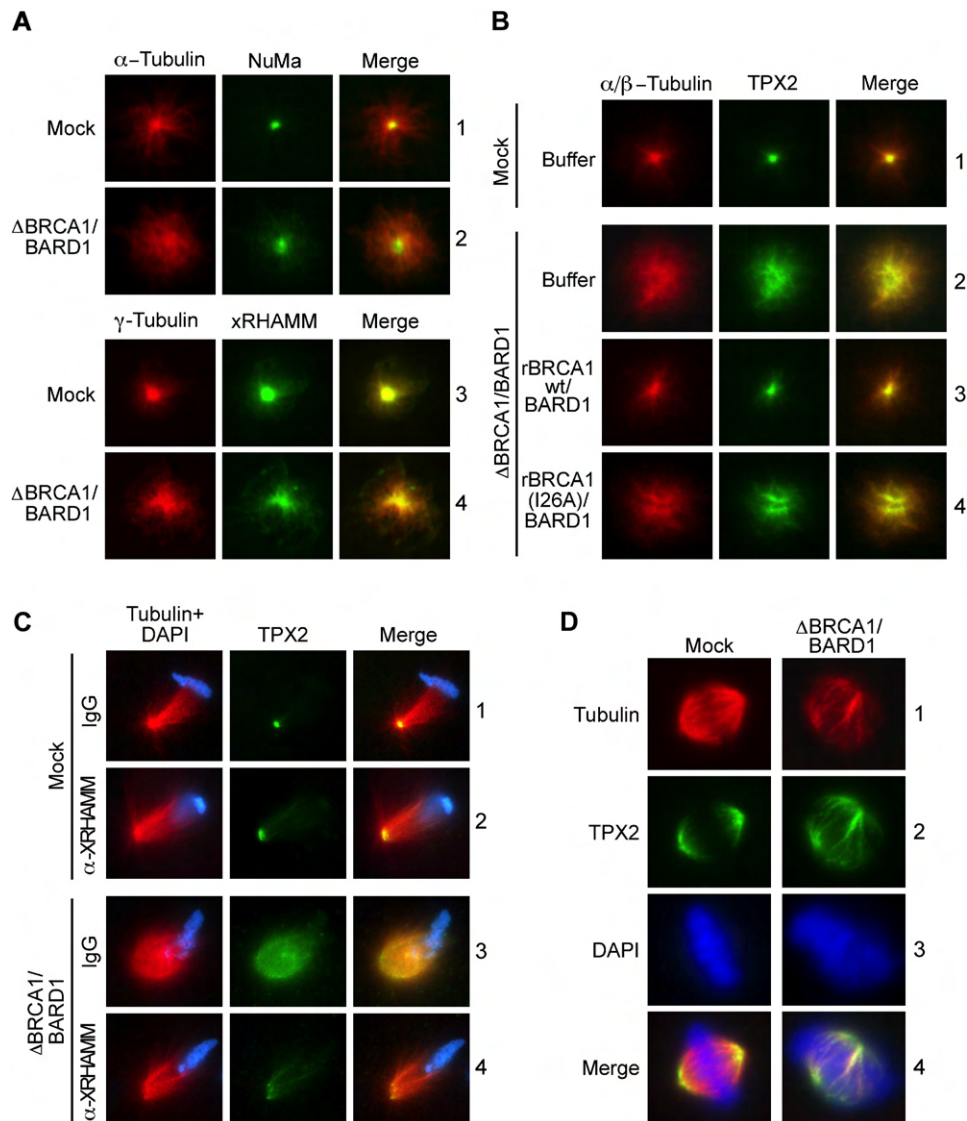


Figure 5. BRCA1/BARD1 Is Required for Concentrating TPX2 on Spindle Poles

(A) IF images of Ran-GTP-induced MT asters assembled in mock-treated and BRCA1/BARD1-depleted extracts after methanol fixation followed by staining with antibodies directed against NuMa and α -tubulin (rows 1 and 2) or γ -tubulin and XRHAMM (rows 3 and 4).

(B and C) Representative Ran-GTP-induced (B) and chromatin-induced (C) asters assembled in mock-treated and BRCA1/BARD1-depleted extracts supplemented with rhodamine-labeled tubulin, Alexa Fluor 488-labeled anti-TPX2 Ab, and the indicated components.

(D) Representative IF images of TPX2 localization on metaphase spindles in HeLa cells transfected with control or BRCA1- and BARD1-specific siRNAs.

with TPX2 concentrated at the center of each pole (Figure 5C, row 1). In contrast, in BRCA1/BARD1-depleted extract, chromatin-induced spindles exhibited disorganized poles, and TPX2 diffusely bound to microtubules (Figure 5C, row 3; for more examples, see Figure S4).

We next compared the localization of TPX2 on metaphase spindles of HeLa cells transfected with either control or BRCA1/BARD1-specific siRNAs. In control cells, TPX2 tightly concentrated in the vicinity of spindle poles of all metaphase spindles analyzed. In contrast, in ~20% of BRCA1/BARD1 siRNA-treated metaphase cells, TPX2 was diffusely localized along the length of spindle micro-

tubules and failed to concentrate on spindle poles (Figure 5D, compare panels in row 2).

These observations demonstrate that BRCA1/BARD1 and its E3 ubiquitin ligase activity control spindle-pole assembly by facilitating targeting of TPX2 to spindle poles independent of centrosomes.

BRCA1/BARD1 Associates with Spindle-Pole-Organizing Proteins

To test whether BRCA1/BARD1 associates with spindle-pole-organizing proteins, we analyzed BRCA1 immunoprecipitates (IPs) for the presence of TPX2, NuMa, and

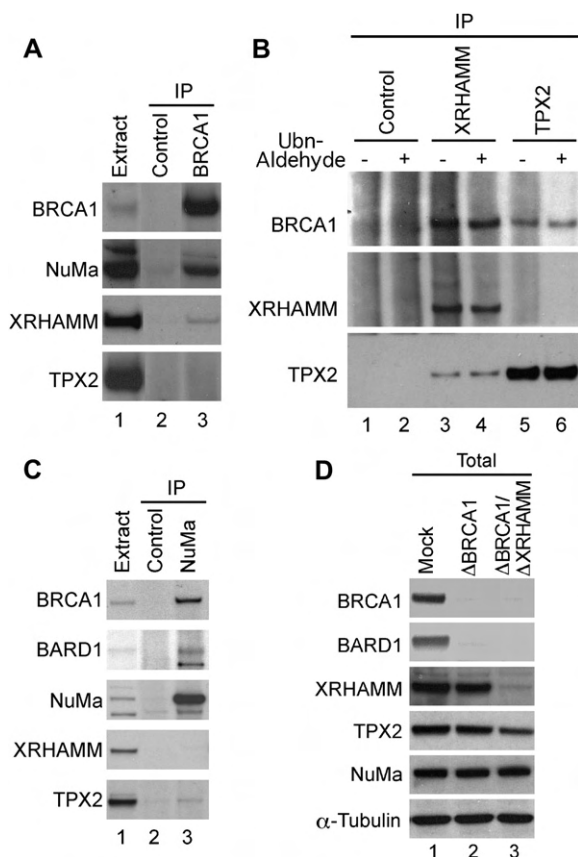


Figure 6. BRCA1/BARD1 Interacts with Spindle-Pole-Organizing Proteins

(A–C) CSF-arrested extracts were incubated for 1 hr at 21°C followed by IP with nonimmune Ig (Control) or the indicated antibodies. The IPs were analyzed by W blotting with the indicated antibodies. Where indicated, the extracts were supplemented with 6 μ M ubiquitin aldehyde (+) prior to incubation (B).

(D) The mock-treated, BRCA1/BARD1-depleted, and BRCA1/BARD1+XRHAMM-codepleted CSF-arrested extracts were analyzed by W blotting with the indicated antibodies.

XRHAMM. Both NuMa and XRHAMM were significantly enriched in these fractions compared to control IPs (Figure 6A, lane 3 versus 2). Although TPX2 was not enriched in anti-BRCA1 IPs (Figure 6A), an identical experiment performed with TPX2 Ab led to specific co-immunoprecipitation of BRCA1/BARD1 (Figure 6B, lanes 5 and 6 versus 1 and 2). An analogous result was obtained with XRHAMM- and NuMa-specific antibodies (Figure 6B, lanes 3 and 4 versus 1 and 2; Figure 6C, lane 3 versus 2).

An association between XRHAMM and TPX2 requires additional factors present in extract (Groen et al., 2004). One could envision that BRCA1/BARD1 regulates the XRHAMM-TPX2 interaction by ubiquitination. However, this scenario seems unlikely since the XRHAMM-TPX2 interaction was not affected by BRCA1/BARD1 depletion (data not shown) or by supplementing an extract with

ubiquitin aldehyde, a potent inhibitor of multiple deubiquitinating enzymes (Figure 6B). Furthermore, BRCA1/BARD1 depletion did not affect the electrophoretic mobility or abundance of XRHAMM, TPX2, NuMa, or γ -tubulin (Figure 6D and data not shown). The latter outcome likely reflects the fact that, in egg extract, BRCA1/BARD1 is considerably less abundant than SAFs and therefore interacts with only a small fraction of these proteins. Indeed, no more than 5% of NuMa or XRHAMM was found to associate with BRCA1/BARD1 (Figure 6A).

Given the specific association of BRCA1/BARD1 with the aforementioned SAFs, binding of BRCA1/BARD1 to microtubules was also tested in extracts and mammalian cells. Using multiple BRCA1- and BARD1-monospecific antibodies, we did not detect clear colocalization of BRCA1 and/or BARD1 with spindle microtubules (Figures 1D and 1E and data not shown). These results demonstrate that BRCA1/BARD1 physically interacts with SAFs participating in the processes of Ran-dependent MT polymerization and spindle-pole assembly (i.e., with NuMa, XRHAMM, and TPX2). Thus far, there is no evidence supporting the notion that BRCA1 and BARD1 are themselves MT-associated proteins (MAPs) or are involved in MAP ubiquitination.

BRCA1/BARD1 Regulates Mitotic MT Organization in a XRHAMM-Dependent Manner

While analyzing the localization of XRHAMM on microtubules, a surprising observation was made. Supplementation of the BRCA1/BARD1-depleted extract with an affinity-purified, fluorochrome-labeled Ab directed against a C-terminal segment of XRHAMM (α -XRHAMM) led to rescue of the MT aster defects (data not shown). Rescue was also achieved by supplementing BRCA1/BARD1-depleted extract with small amounts (5–20 ng/ μ l) of unlabeled α -XRHAMM, but not with nonimmune rabbit IgG (Figure 7B, column 2 versus 1). An identical concentration of α -XRHAMM had no effect on MT asters in the mock-treated extract (Figure 7A, column 2 versus 1). Moreover, MT aster assembly was not affected when either mock-treated or BRCA1/BARD1-depleted extracts were supplemented with similar amounts of Ab directed against NuMa or TPX2 (data not shown). α -XRHAMM (20 ng/ μ l) also efficiently rescued defects in asters and spindles assembled around sperm chromatin in BRCA/BARD1-depleted extract (Figure 5C, row 4 versus 3).

To elucidate the functions of the region of XRHAMM that is targeted by α -XRHAMM, we added to extracts a C-terminal fragment of XRHAMM (aa 1038–1175; XRHAMM-CWT) that had been used as immunogen during Ab development. Interestingly, on its own, XRHAMM-CWT (1.25–4 μ M) disrupted aster-pole structure and prevented efficient TPX2 accumulation on aster poles in mock-treated extract (Figure 7A, column 3 versus 1). Surprisingly, addition of this peptide to the BRCA1/BARD1-depleted extract had an even more dramatic effect. MT asters became severely disorganized, and TPX2 was abnormally bound along the length of thick, disoriented MT fibers (Figure 7B, column 3

versus 1). XRHAMM-CWT contains a leucine zipper motif, and its amino acid sequence exhibits high interspecies conservation (Groen et al., 2004; Maxwell et al., 2003). This XRHAMM motif appears to be functionally important because a C-terminal fragment in which three conserved leucines of the leucine zipper were replaced by arginines (XRHAMM-CR³) was inactive in disrupting aster structure and TPX2 targeting to aster poles (Figure 7A, column 4 versus 3). It also failed to generate the “extreme” aster phenotype produced by the WT peptide in BRCA1/BARD1-depleted extract (Figure 7B, column 4 versus 3). Nevertheless, XRHAMM-CR³ retained the ability to bind α -XRHAMM as efficiently as XRHAMM-CWT (Figure 7C, lane 5 versus 4). Indeed, when it had fully titrated the available α -XRHAMM, it abrogated the rescue effect of this Ab in BRCA1/BARD1-depleted extract (Figure 7B, column 5 versus 2). This result indicates that the effect of the Ab on aster structure is specific to XRHAMM.

These experiments revealed that α -XRHAMM and the recombinant peptide fragment against which it was generated exhibited opposite effects on MT asters in BRCA1/BARD1-depleted extract: The Ab rescued, while the peptide aggravated, the MT aster phenotype (Table S1). Two interpretations of these results were considered: (1) XRHAMM function is inhibited in the absence of BRCA1/BARD1; the Ab stimulates, while the peptide further inhibits XRHAMM function, or (2) XRHAMM is hyperactive in the absence of BRCA1/BARD1; the Ab downregulates XRHAMM function, and the peptide activates it.

To distinguish between these possibilities, we compared the effects of adding α -XRHAMM and XRHAMM-CWT on the structure of asters and TPX2 MT localization in an extract that had been partially depleted of XRHAMM. Depleting extract of XRHAMM by 90%–95% significantly inhibited both the abundance and size of asters (Figures 7D and 7E, column 2 versus 1). Although XRHAMM was previously shown to be required for TPX2 concentration on spindle poles (Groen et al., 2004), in this setting, TPX2 still concentrated at the centers of faint MT asters, suggesting that the amount of residual XRHAMM in the extract (5% to 10%) was sufficient to perform certain key XRHAMM functions, albeit inefficiently. In keeping with this notion, the effect of adding α -XRHAMM to this extract was additive with XRHAMM depletion—i.e., the Ab further decreased the efficiency of aster formation as well as the intensity of tubulin and TPX2 staining at aster centers (Figures 7D and 7E, column 3 versus 2). By contrast, addition of XRHAMM-CWT to the extract, which was partially depleted of XRHAMM, led to a substantial rescue of the aster formation defect: The asters were larger and TPX2 was concentrated on aster centers, although in a somewhat more diffuse manner than in asters assembled in mock-treated extract (Figure 7E, column 4 versus 2). The peptide, however, failed to significantly increase the abundance of asters formed (Figure 7D). These results indicate that α -XRHAMM inhibits XRHAMM MT-organizing function, while XRHAMM-CWT can partially compensate for the loss of XRHAMM function.

Because abnormalities associated with BRCA1/BARD1 depletion were rescued by α -XRHAMM and because this reagent appears to inhibit XRHAMM function, the data suggest that XRHAMM is hyperactive in the absence of BRCA1/BARD1, a condition that is deleterious for spindle function. As a further test of this hypothesis, we asked whether the spindle-pole defects caused by BRCA1/BARD1 depletion could be rescued by partial elimination of XRHAMM from extract. Such treatment indeed rescued aster architecture and localization of TPX2 on aster poles to an extent similar to addition of Ab (Figure 7F, column 4 versus columns 2 and 3). Results of the experiments involving α -XRHAMM and XRHAMM-CWT are summarized in Table S1.

Taken together, these observations indicate that BRCA1/BARD1 contributes to proper spindle assembly by attenuating the otherwise excessive activity of XRHAMM in mitosis.

DISCUSSION

BRCA1/BARD1 Controls Ran-Dependent Mitotic Spindle Assembly

This study demonstrates a critical role for BRCA1/BARD1 in mitotic MT organization and spindle-pole assembly in both *Xenopus* egg extracts and cultured mammalian cells. Spindle poles form by concentrating MT minus ends at their centers, a process that does not require centrosomes but rather relies on the activity of various noncentrosomal MAPs as well as plus- and minus-end-directed motor proteins (reviewed in Fant et al., 2004). Two MAPs critical for spindle-pole assembly, NuMA and TPX2, are transported to spindle poles by the minus-end-directed motor complex, dynein/dynactin (Merdes et al., 2000; Wittmann et al., 2000). NuMA remained at the centers of unfocused aster poles that assembled in BRCA1/BARD1-depleted extract, suggesting that BRCA1/BARD1 is not required for the dynein/dynactin-dependent transport per se. TPX2, however, was not effectively targeted to spindle poles in this setting, providing evidence for a specific line of communication between BRCA1/BARD1 and a key step in spindle-pole formation.

Despite spindle abnormalities, BRCA1/BARD1-deficient cells exited mitosis, in keeping with the previously reported spindle-assembly checkpoint defect in cells expressing a hypomorphic mutant *BRCA1* allele (Wang et al., 2004; Xu et al., 1999). However, the fidelity of mitotic exit was compromised, as evidenced by the appearance of chromosome segregation defects and micronucleus formation in BRCA1/BARD1-siRNA-treated cells (Figure 3E) and the postmitotic nuclear-assembly defect in BRCA1/BARD1-depleted egg extract (Figure 2C and Figure S1A). Of note, multiple nuclei and micronuclei were previously observed in BRCA1-deficient cells, and this defect was thought to result from multipolar spindle formation due to abnormal centrosome amplification in these cells (Xu et al., 1999).

We speculate that both spindle-pole abnormalities and postmitotic nuclear-assembly defects that develop in

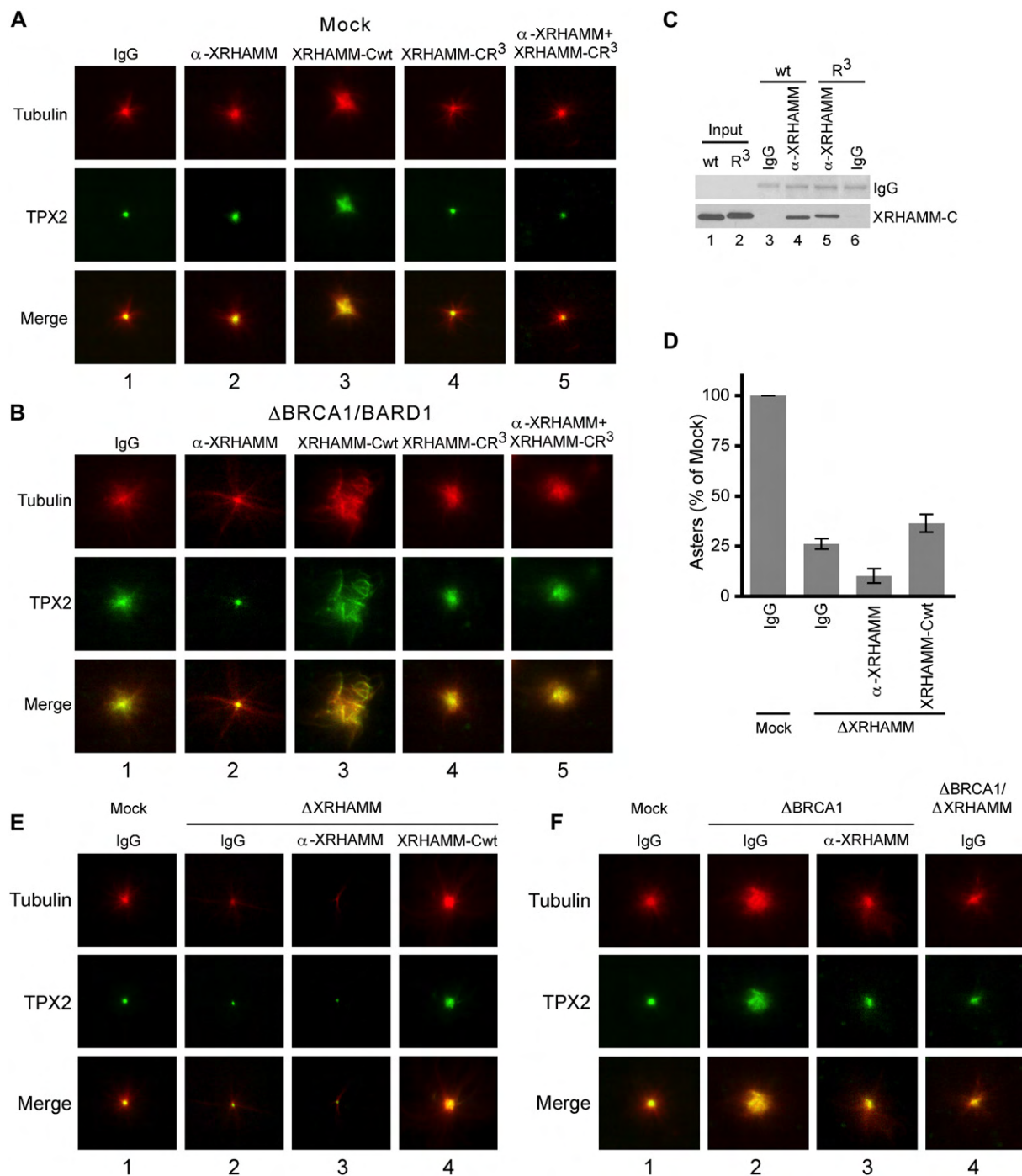


Figure 7. BRCA1/BARD1 Regulates MT Organization in a XRHAMM-Dependent Fashion

Prior to assaying, egg extracts were supplemented with rhodamine-labeled tubulin and Alexa Fluor 488-labeled anti-TPX2 Ab; MT asters were induced by the addition of Ran(Q69L)-GTP (except in [C]).

(A and B) Representative asters assembled in mock-treated (A) and BRCA1/BARD1-depleted (B) extracts supplemented with the indicated components.

(C) Disruption of its leucine zipper does not affect XRHAMM-C interaction with the corresponding specific Ab. Equal amounts of recombinant XRHAMM-CWT (wt) and XRHAMM-CR³ (R³) were immunoprecipitated with the affinity-purified α -XRHAMM (lanes 4 and 5, respectively) or nonimmune IgG (lanes 3 and 6, respectively) and analyzed by SDS-PAGE followed by staining of the membrane with Ponceau S (IgG) or S protein-HRP (XRHAMM-C). Note that XRHAMM-CR³ exhibits slightly slower electrophoretic mobility than XRHAMM-CWT.

(D and E) Quantitative analysis of asters (D) and representative structures (E) assembled in the mock-treated and XRHAMM-depleted extracts supplemented with the indicated components. Values in (D) represent means \pm standard deviations of two independent measurements.

BRCA1/BARD1-deficient settings can be attributed to deregulation of TPX2. In this regard, Ran-GTP and its target proteins (and BRCA1/BARD1 partners) TPX2 and NuMA play a role in both spindle formation/function and post-mitotic nuclear assembly (Compton and Cleveland, 1993; Hetzer et al., 2002; Merdes and Cleveland, 1998; O'Brien and Wiese, 2006). Spindle-pole disorganization due to mitotic TPX2 dysfunction may lead to inefficient chromosome tethering to spindle poles in anaphase/telophase, followed by enclosure of the resulting loose/lagging chromosomes by the nuclear envelope. Such a mechanism has been proposed as the reason for similar abnormalities seen in cells with perturbed NuMA function (Merdes and Cleveland, 1998). In addition, TPX2 has been implicated in nuclear assembly and nuclear-envelope growth independent of its mitotic function (O'Brien and Wiese, 2006). The evidence for a biochemical interaction of BRCA1/BARD1 with TPX2, XRHAMM, and NuMA in egg extract supports the existence of a functional link between these proteins. Moreover, in human cell extracts, BRCA1/BARD1 also coexists in a complex with NuMA (R. Greenberg, B. Sobhian, and D.M.L., unpublished data). Thus, in both mammalian cells and frog egg extracts, there is evidence for BRCA1/BARD1 interacting with spindle-pole constituents.

One wonders whether NuMA and TPX2, which, like BRCA1/BARD1, are localized in the interphase nucleus (Compton and Cleveland, 1993; Wittmann et al., 2000), also engage in certain postdamage S phase functions of BRCA1/BARD1 (e.g., repair of double-strand breaks, checkpoint activation, etc.). In this regard, there is a growing list of proteins, including members of the Ran pathway, that perform seemingly unrelated functions during mitosis and interphase (reviewed in Hetzer et al., 2005; Quimby and Dasso, 2003).

BRCA1/BARD1 Regulates the MT-Organizing Function of XRHAMM

This study demonstrates that BRCA1/BARD1 facilitates TPX2 targeting to spindle poles by downmodulating XRHAMM function. Data presented here also implicate the highly conserved, leucine-zipper-bearing C-terminal domain of XRHAMM as a contributor to those aspects of MT-organizing function that are regulated by BRCA1/BARD1. Given that the leucine zipper is a potential protein-interacting motif, we speculate that the C-terminal domain of XRHAMM is involved in the formation of XRHAMM homodimers or heterodimers with other SAFs and that these interactions are important for targeting of TPX2 to spindle poles. In a similar vein, one way of explaining the XRHAMM-agonistic effect of XRHAMM-CWT is to propose that it interferes with the homo- and/or heterodimerization of endogenous XRHAMM.

The E3 ubiquitin ligase activity of BRCA1/BARD1 appears to be involved in its MT-organizing function because an enzymatically deficient heterodimer, rBRCA1(I26A)/BARD1, was considerably less efficient than its WT counterpart in reversing an abnormal aster phenotype. Conceivably, BRCA1/BARD1 ubiquitinates TPX2, XRHAMM, and/or NuMA, and this modification is required for the targeting of TPX2 to spindle poles. If BRCA1/BARD1 does ubiquitinate any of these proteins, the modification might be transient and/or sensitive to the action of certain deubiquitinating enzymes, one of which, BAP1, is known to associate with BRCA1/BARD1 (Jensen et al., 1998). Alternatively, BRCA1/BARD1 might regulate XRHAMM/TPX2 indirectly, via ubiquitination of additional protein partners. Further studies addressing the functional link between BRCA1/BARD1 and XRHAMM/TPX2 will be essential for understanding the mechanisms of spindle-pole assembly.

A New Pathway for BRCA1-Mediated Tumor Suppression?

There are reasons to believe that the newly uncovered function of BRCA1/BARD1 in the control of Ran-dependent MT and spindle-pole assembly is related to the acknowledged role of BRCA1 in the maintenance of genome stability and tumor suppression. Failure to properly form spindle poles compromises the mechanical integrity of the spindle apparatus and can lead to chromosome segregation defects and aneuploidy, abnormalities that are characteristic of both BRCA1/BARD1-deficient cells and many tumor cells (Fant et al., 2004; Xu et al., 1999). Furthermore, our study implicates BRCA1 in the regulation of SAFs that have been previously linked to cancer in their own right (Figure S5). Aberrant expression of RHAMM and TPX2, as well as Aurora A, a mitotic kinase whose localization and activity are regulated by TPX2 (reviewed in Crane et al., 2003), were linked to malignant transformation as well as progression of certain human tumors (Crane et al., 2003; Maxwell et al., 2005; Smith et al., 2006 and references therein). Moreover, RHAMM and TPX2 are considered candidate oncoproteins (Hall et al., 1995; Maxwell et al., 2005; Smith et al., 2006), and Aurora A is a likely oncoprotein given that its gene is amplified and its mRNA is overexpressed in multiple human cancers. Furthermore, ectopic overexpression of Aurora A is sufficient to transform certain cell types (reviewed in Crane et al., 2003). In this regard, we have also found that TPX2 mislocalization in BRCA1-deficient cells leads to mislocalization of Aurora A (data not shown).

In keeping with our observations, a recent independent study based on a "breast cancer network" model of mammalian functional genomic and protein interaction parameters has suggested a functional link between

(F) Representative asters assembled in the mock-treated (column 1), BRCA1/BARD1-depleted (columns 2 and 3), or BRCA1/BARD1 + XRHAMM-codepleted (column 4) extracts supplemented with the indicated components. W blots of the corresponding extracts are shown in Figure 6D.

BRCA1, RHAMM, and Aurora A (M.A. Pujana et al., unpublished data). In addition, Aurora A has been shown to phosphorylate both TPX2 and BRCA1, suggesting a possible feedback connection between this kinase and its regulators (Crane et al., 2003; Ouchi et al., 2004). Whether certain aspects of a *BRCA1*^{-/-} breast or ovarian cancer phenotype are a product of dysfunction of XRHAMM, TPX2, and/or Aurora A remains to be determined.

Finally, another breast and ovarian tumor suppressor, BRCA2, which interacts physically with BRCA1, has recently been implicated in the control of cytokinesis (Daniels et al., 2004; Venkitaraman, 2002). It will be interesting to learn whether the cytokinesis function of BRCA2 is related to the BRCA1/BARD1 mitotic/MT-organizing function and, if so, whether a defect in this complex set of events contributes to a breakdown in BRCA1 and/or BRCA2 tumor-suppression function.

EXPERIMENTAL PROCEDURES

Recombinant Proteins and Antibodies

The recombinant FLAG-BRCA1/HA-BARD1 heterodimers were produced using the Bac-to-Bac Baculovirus Expression System (Gibco BRL) and doubly immunoaffinity purified prior to use. In vitro analysis of *Xenopus* BRCA1/BARD1 E3 ligase activity was carried out as previously described for the human heterodimer (Mallery et al., 2002). Plasmid construction, protein expression and purification, and antibodies used in this study are described in the Supplemental Experimental Procedures.

Xenopus Egg Extracts

Crude egg extracts were prepared as described (Murray, 1991). Metaphase extracts were released into interphase by addition of 0.5 mM CaCl₂. Immunodepletions were carried out using specific antibodies bound to protein A-Sepharose. Incubations of extracts were carried out at 21°C unless indicated otherwise. For more details on extracts and immunodepletions, see the Supplemental Experimental Procedures.

Analysis of Chromatin, Mitotic Spindles, and Asters in Egg Extracts

DNA replication was analyzed by measuring the incorporation of [α -³²P]dATP into DNA as described (Dasso and Newport, 1990). Analysis of chromatin in egg extract is detailed in the Supplemental Experimental Procedures. Metaphase bipolar spindles were assembled in egg extract supplemented with 75 μ g/ml of rhodamine tubulin (Cytoskeleton) as described (Desai et al., 1999). MT asters were induced by supplementing CSF-arrested extract with 15 μ M Ran(Q69L)-GTP. For IF analysis of NuMA, XRHAMM, and γ -tubulin localization, MT spindle and aster structures were isolated by centrifugation through a glycerol cushion, fixed, and stained with the corresponding antibodies as described (Desai et al., 1999). TPX2 localization on MT asters and spindles was analyzed by direct IF microscopy of extracts supplemented with anti-TPX2 Ab (5 ng/ μ l). The antibody was labeled with Alexa Fluor 488 carboxylic acid, succinimidyl ester (Groen et al., 2004).

Immunoprecipitations

Immunoprecipitations from *Xenopus* egg extracts were performed as detailed in the Supplemental Experimental Procedures.

Analysis of BRCA1/BARD1 Mitotic Function in HeLa Cells

HeLa cells were cultivated in DMEM/10% fetal calf serum. Cells were seeded on coverslips in a 6-well plate and were transfected 24 hr later

with a mixture of hBRCA1 and hBARD1 SMARTpool siRNAs (100 nM each) or with an equal amount of the control nontargeting siRNAs (Dharmacon). Transfections were carried out using Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four hours after the first transfection, a second, identical, transfection was carried out. Thirty-six hours after the second transfection, cells were washed in PBS, fixed in methanol/acetone (7:3 mixture) at -20°C, and immunostained. Alternatively, cells were permeabilized with digitonin and fixed with formaldehyde as described (Joseph et al., 2002). Coverslips were mounted over DAPI-containing VECTASHIELD stain (Vector Laboratories).

Fluorescence Microscopy and Image Analysis

Fluorescence microscopy of chromatin and spindle structures in egg extract was carried out using an Eclipse E600 (Nikon) equipped with a SPOT camera (Diagnostic Instruments) and an Axioskop 2 (Zeiss). Fluorescence microscopy of HeLa cells was performed using the Axioskop 2. Images were obtained and analyzed using Spot RT Software v3.0 (Diagnostic Instruments) and AxioVision software (Zeiss). Three-dimensional surface plots of MT asters were generated using the program, ImageJ 1.34s (<http://rsb.info.nih.gov/ij/>).

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, Supplemental References, five figures, and one table and can be found with this article online at <http://www.cell.com/cgi/content/full/127/3/539/DC1/>.

ACKNOWLEDGMENTS

We thank M. Dasso, T. Hirano, D. Compton, and T. Mitchison for generous gifts of reagents and R. Ohi, J.S. Stanford, E. Arias, R. Greenberg, B. Sobhian, S. Ganesan, A. DeNicolo, and other members of the Livingston, Walter, and Mitchison laboratories for many helpful discussions and for reagents. We also wish to thank J.B.A. Green for considerable advice and insight and W. Luo and R.S. Gelman for statistical analysis of the data. We apologize to authors whose work could not be directly cited owing to space constraints. This work was supported by grants from the National Cancer Institute to D.M.L.; a Stewart Trust grant, NIH grant GM62267, and ACS grant 106201 to J.C.W., and Department of Defense Breast Cancer Research Program award W81XWH-04-1-0524 to V.J. D.M.L. is a grantee of and consultant to the Novartis Institute for Biomedical Research.

Received: March 28, 2005

Revised: June 23, 2006

Accepted: August 31, 2006

Published: November 2, 2006

REFERENCES

- Boulton, S.J., Martin, J.S., Polanowska, J., Hill, D.E., Gartner, A., and Vidal, M. (2004). BRCA1/BARD1 orthologs required for DNA repair in *Caenorhabditis elegans*. *Curr. Biol.* 14, 33–39.
- Brzovic, P.S., Keefe, J.R., Nishikawa, H., Miyamoto, K., Fox, D., 3rd, Fukuda, M., Ohta, T., and Klevit, R. (2003). Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-higase complex. *Proc. Natl. Acad. Sci. USA* 100, 5646–5651.
- Carazo-Salas, R.E., Guarguaglini, G., Gruss, O.J., Segref, A., Karsenti, E., and Mattaj, I.W. (1999). Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. *Nature* 400, 178–181.
- Choudhury, A.D., Xu, H., and Baer, R. (2004). Ubiquitination and proteasomal degradation of the BRCA1 tumor suppressor is regulated during cell cycle progression. *J. Biol. Chem.* 279, 33909–33918.

- Compton, D.A., and Cleveland, D.W. (1993). NuMA is required for the proper completion of mitosis. *J. Cell Biol.* 120, 947–957.
- Crane, R., Gadea, B., Littlepage, L., Wu, H., and Ruderman, J.V. (2003). Aurora A, meiosis and mitosis. *Biol. Cell.* 96, 215–229.
- Daniels, M.J., Wang, Y., Lee, M., and Venkitaraman, A.R. (2004). Abnormal cytokinesis in cells deficient in the breast cancer susceptibility protein BRCA2. *Science* 306, 876–879.
- Dasso, M., and Newport, J.W. (1990). Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in *Xenopus*. *Cell* 61, 811–823.
- Deng, C.X., and Wang, R.H. (2003). Roles of BRCA1 in DNA damage repair: a link between development and cancer. *Hum. Mol. Genet.* 12, R113–R123.
- Desai, A., Murray, A., Mitchison, T.J., and Walczak, C.E. (1999). The use of *Xenopus* egg extracts to study mitotic spindle assembly and function in vitro. *Methods Cell Biol.* 61, 385–412.
- Fang, S., Lorick, K.L., Jensen, J.P., and Weissman, A.M. (2003). RING finger ubiquitin protein ligases: implications for tumorigenesis, metastasis and for molecular targets in cancer. *Semin. Cancer Biol.* 13, 5–14.
- Fant, X., Merdes, A., and Haren, L. (2004). Cell and molecular biology of spindle poles and NuMA. *Int. Rev. Cytol.* 238, 1–57.
- Glover, J.N., Williams, R.S., and Lee, M.S. (2004). Interactions between BRCT repeats and phosphoproteins: tangled up in two. *Trends Biochem. Sci.* 29, 579–585.
- Groen, A.C., Cameron, L.A., Coughlin, M., Miyamoto, D.T., Mitchison, T.J., and Ohi, R. (2004). XRHAMM functions in ran-dependent microtubule nucleation and pole formation during anastral spindle assembly. *Curr. Biol.* 14, 1801–1811.
- Hall, C.L., Yang, B., Yang, X., Zhang, S., Turley, M., Samuel, S., Lange, L.A., Wang, C., Curpen, G.D., Savani, R.C., et al. (1995). Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-ras transformation. *Cell* 82, 19–26.
- Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H., and Ohta, T. (2001). The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J. Biol. Chem.* 276, 14537–14540.
- Hetzer, M., Gruss, O.J., and Mattaj, I.W. (2002). The Ran GTPase as a marker of chromosome position in spindle formation and nuclear envelope assembly. *Nat. Cell Biol.* 4, E177–E184.
- Hetzer, M.W., Walther, T.C., and Mattaj, I.W. (2005). Pushing the envelope: structure, function, and dynamics of the nuclear periphery. *Annu. Rev. Cell Dev. Biol.* 21, 347–380.
- Hsu, L.C., and White, R.L. (1998). BRCA1 is associated with the centrosome during mitosis. *Proc. Natl. Acad. Sci. USA* 95, 12983–12988.
- Jensen, D.E., Proctor, M., Marquis, S.T., Gardner, H.P., Ha, S.I., Chodosh, L.A., Ishov, A.M., Tommerup, N., Vissing, H., Sekido, Y., et al. (1998). BAP1: a novel ubiquitin hydrolase which binds to the BRCA1 RING finger and enhances BRCA1-mediated cell growth suppression. *Oncogene* 16, 1097–1112.
- Joseph, J., Tan, S.H., Karpova, T.S., McNally, J.G., and Dasso, M. (2002). SUMO-1 targets RanGAP1 to kinetochores and mitotic spindles. *J. Cell Biol.* 156, 595–602.
- Joukov, V., Chen, J., Fox, E.A., Green, J.B., and Livingston, D.M. (2001). Functional communication between endogenous BRCA1 and its partner, BARD1, during *Xenopus laevis* development. *Proc. Natl. Acad. Sci. USA* 98, 12078–12083.
- Ludwig, T., Chapman, D.L., Papaioannou, V.E., and Efstratiadis, A. (1997). Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of *Brca1*, *Brca2*, *Brca1/Brca2*, *Brca1/p53*, and *Brca2/p53* nullizygous embryos. *Genes Dev.* 11, 1226–1241.
- Mallery, D.L., Vandenberg, C.J., and Hiom, K. (2002). Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *EMBO J.* 21, 6755–6762.
- Maxwell, C.A., Keats, J.J., Crainie, M., Sun, X., Yen, T., Shibuya, E., Hendzel, M., Chan, G., and Pilarski, L.M. (2003). RHAMM is a centrosomal protein that interacts with dynein and maintains spindle pole stability. *Mol. Biol. Cell* 14, 2262–2276.
- Maxwell, C.A., Keats, J.J., Belch, A.R., Pilarski, L.M., and Reiman, T. (2005). Receptor for hyaluronan-mediated motility correlates with centrosome abnormalities in multiple myeloma and maintains mitotic integrity. *Cancer Res.* 65, 850–860.
- McCarthy, E.E., Celebi, J.T., Baer, R., and Ludwig, T. (2003). Loss of Bard1, the heterodimeric partner of the Brca1 tumor suppressor, results in early embryonic lethality and chromosomal instability. *Mol. Cell. Biol.* 23, 5056–5063.
- Merdes, A., and Cleveland, D.W. (1998). The role of NuMA in the interphase nucleus. *J. Cell Sci.* 111, 71–79.
- Merdes, A., Heald, R., Samejima, K., Earnshaw, W.C., and Cleveland, D.W. (2000). Formation of spindle poles by dynein/dynactin-dependent transport of NuMA. *J. Cell Biol.* 149, 851–862.
- Miki, Y., Swensen, J., Shattuck-Eidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q., Cochran, C., Bennett, L.M., and Ding, W. (1994). A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science* 266, 66–71.
- Moore, W., Zhang, C., and Clarke, P.R. (2002). Targeting of RCC1 to chromosomes is required for proper mitotic spindle assembly in human cells. *Curr. Biol.* 12, 1442–1447.
- Murray, A.W. (1991). Cell cycle extracts. *Methods Cell Biol.* 36, 581–605.
- O'Brien, L.L., and Wiese, C. (2006). TPX2 is required for postmitotic nuclear assembly in cell-free *Xenopus laevis* egg extracts. *J. Cell Biol.* 173, 685–694.
- Ouchi, M., Fujiuchi, N., Sasai, K., Katayama, H., Minamishima, Y.A., Ongusaha, P.P., Deng, C., Sen, S., Lee, S.W., and Ouchi, T. (2004). BRCA1 phosphorylation by Aurora-A in the regulation of G2 to M transition. *J. Biol. Chem.* 279, 19643–19648.
- Quimby, B.B., and Dasso, M. (2003). The small GTPase Ran: interpreting the signs. *Curr. Opin. Cell Biol.* 15, 338–344.
- Sato, K., Hayami, R., Wu, W., Nishikawa, T., Nishikawa, H., Okuda, Y., Ogata, H., Fukuda, M., and Ohta, T. (2004). Nucleophosmin/B23 is a candidate substrate for the BRCA1-BARD1 ubiquitin ligase. *J. Biol. Chem.* 279, 30919–30922.
- Scully, R., Chen, J., Plug, A., Xiao, Y., Weaver, D., Feunteun, J., Ashley, T., and Livingston, D.M. (1997). Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* 88, 265–275.
- Smith, L.T., Mayerson, J., Nowak, N.J., Suster, D., Mohammed, N., Long, S., Auer, H., Jones, S., McKeegan, C., Young, G., et al. (2006). 20q11.1 amplification in giant-cell tumor of bone: Array CGH, FISH, and association with outcome. *Genes Chromosomes Cancer* 45, 957–966.
- Starita, L.M., Machida, Y., Sankaran, S., Elias, J.E., Griffin, K., Schlegel, B.P., Gygi, S.P., and Parvin, J.D. (2004). BRCA1-dependent ubiquitination of gamma-tubulin regulates centrosome number. *Mol. Cell. Biol.* 24, 8457–8466.
- Venkitaraman, A.R. (2002). Cancer susceptibility and the functions of BRCA1 and BRCA2. *Cell* 108, 171–182.
- Wang, R.H., Yu, H., and Deng, C.X. (2004). A requirement for breast-cancer-associated gene 1 (*BRCA1*) in the spindle checkpoint. *Proc. Natl. Acad. Sci. USA* 101, 17108–17113.
- Wilde, A., and Zheng, Y. (1999). Stimulation of microtubule aster formation and spindle assembly by the small GTPase Ran. *Science* 284, 1359–1362.
- Wittmann, T., Wilm, M., Karsenti, E., and Vernos, I. (2000). TPX2, a novel *Xenopus* MAP involved in spindle pole organization. *J. Cell Biol.* 149, 1405–1418.

Wu, L.C., Wang, Z.W., Tsan, J.T., Spillman, M.A., Phung, A., Xu, X.L., Yang, M.C., Hwang, L.Y., Bowcock, A.M., and Baer, R. (1996). Identification of a RING protein that can interact in vivo with the *BRCA1* gene product. *Nat. Genet.* *14*, 430–440.

Xu, X., Weaver, Z., Linke, S.P., Li, C., Gotay, J., Wang, X.W., Harris, C.C., Ried, T., and Deng, C.X. (1999). Centrosome amplifi-

cation and a defective G2-M cell cycle checkpoint induce genetic instability in *BRCA1* exon 11 isoform-deficient cells. *Mol. Cell* *3*, 389–395.

Yu, X., Fu, S., Lai, M., Baer, R., and Chen, J. (2006). *BRCA1* ubiquitinates its phosphorylation-dependent binding partner CtIP. *Genes Dev.* *20*, 1721–1726.

SUPPLEMENTAL DATA

SUPPLEMENTAL EXPERIMENTS

Postmitotic nuclear assembly defect in BRCA/BARD1-depleted CSF-arrested extract, upon release from the arrest (See Figure S1)

When sperm chromatin was mixed with BRCA1/BARD1-depleted CSF extract that had been released into interphase with Ca^{2+} , chromatin decondensation, formation of nuclei, and rate of DNA replication were unaffected compared to mock-depleted extract (Figures S1A, 'Interphase' and data not shown). When sperm chromatin was added to the same mock-treated or BRCA1-BARD1-depleted CSF extracts that maintained the metaphase arrest, chromatin condensation in both extracts occurred with similar kinetics (Figure S1A, 'Mitosis', and data not shown). We noticed, however, the appearance of scattered mitotic chromosomes in the BRCA1/BARD1-depleted extract, while chromosomes in mock-treated extract were held together (Figure S1A, 'Mitosis', compare panels 'Hoechst'). When extracts containing mitotically condensed chromatin were subsequently driven into interphase by the addition of Ca^{2+} , chromatin in mock-treated extract rapidly decondensed and full-grown, uniform, nuclei were assembled (Figure S1A, 'Mitosis→Interphase', left panels, '60 min' and '90 min'). In contrast, and consistent with what was observed in cycling extracts (Figure 2C), nuclei that formed in BRCA1/BARD1-depleted

extract varied in size, being 3 to 10 times smaller than control nuclei (Figure S1A, 'Mitosis→Interphase', right panels, '60 min' and '90 min').

The postmitotic nuclear assembly defect did not appear to be the result of abnormal mitotic exit in BRCA1/BARD1-depleted extract due to checkpoint activation as evidenced by the fact that inactivation of histone H1 kinase activity occurred with similar kinetics in both immunodepleted and mock-treated extracts (Figure S1B). Moreover, similar defects were detected at very low chromatin concentrations (e.g. 100 sperm nuclei/ μ l), which are insufficient to activate checkpoints ((Minshull, et al., 1994; Murray, 1991) and data not shown). Consistent with these observations, cell cycle-dependent loading and unloading of condensin (XCAP-E) and topoisomerase II, two major reporters of the state of chromatin condensation, occurred with nearly identical kinetics in both mock-treated and BRCA1/BARD1-depleted extracts (Data not shown).

To confirm that the nuclear assembly defect observed in BRCA1/BARD1-depleted extract was a specific outcome of eliminating BRCA1/BARD1, we tested whether this defect could be rescued by supplementing the extract with purified, recombinant *Xenopus* BRCA1/BARD1 heterodimer (rBRCA1/BARD1). When added to BRCA1/BARD1-depleted CSF-arrested extract, the recombinant heterodimer failed to rescue nuclear assembly after Ca^{2+} addition (data not shown). We speculated that rBRCA1/BARD1 lacks essential posttranslational modifications, which can only be added when the protein is sequestered in the nucleus. Thus, chromatin was 'passed' through mock-depleted or BRCA1/BARD1-depleted interphase

extract supplemented with buffer or rBRCA1/BARD1 and, after 90 min, when nuclei had formed, mitosis was induced by the addition of the appropriate control or BRCA1/BARD1-depleted, CSF-arrested, extract. After 1-hour further incubation, each sample was driven back into interphase by the addition of 0.5 mM Ca^{2+} and analyzed for its ability to form nuclei. As in the previous experiments, the BRCA1/BARD1-depleted extract exhibited a postmitotic nuclear assembly defect, and this defect was significantly reversed by the wt rBRCA1/BARD1 (Figures S1C and S1D). Thus, the postmitotic nuclear assembly defect is a specific outcome of BRCA1/BARD1 depletion.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Recombinant proteins and antibodies

The 5' termini of the full-length XBRCA1 and XBARD1 cDNAs (Joukov, et al., 2001) were modified by PCR to bear in frame the FLAG- and the HA epitope tag sequences, respectively. These elements were then subcloned into a pFASTBAC1 vector (Gibco BRL). The I26A mutation was introduced into the aforementioned XBRCA1 cDNA-encoding construct using the QuickChange site-directed mutagenesis kit (Stratagene). The resulting constructs were used to generate the corresponding recombinant baculoviruses in SF21 cells using the Bac-to-BacTM Baculovirus Expression Systems (Gibco BRL). XBRCA1/XBARD1 heterodimer was produced by co-infecting High FiveTM cells

with the corresponding XBRC A1 and XBARD1 baculoviruses. The heterodimer was purified from cell lysates by sequential affinity chromatography using anti-FLAG M2[®] Affinity Gel (Sigma) and anti-HA (12CA5) Sepharose (Ikura, et al., 2000). The eluted heterodimer was dialyzed against buffer A containing 20 mM HEPES (pH 7.6), 100 mM KCl, 2.5 mM MgCl₂, 250 mM sucrose, 1 mM DTT.

Human Ran cDNA was a generous gift of M. Dasso. The cDNA was PCR amplified and inserted in frame into a pET29a(+) vector that encodes N-terminal 6xHis-tag and S-tag sequences (Novagen). The Q69L mutation was introduced using the QuickChange site-directed mutagenesis kit (Stratagene). The fusion proteins were expressed in BL21 Star (DE3) bacterial cells (Invitrogen), purified using Ni-NTA Agarose (Qiagen), and dialyzed against egg lysis buffer (ELB) (10mM HEPES (pH 7.7), 50 mM KCl, 2.5 mM MgCl₂, 1 mM DTT, 250 mM sucrose). Ran wt and Ran(Q69L) were loaded with GTP as previously described (Dasso, et al., 1994). The XRHAMM-Cwt cDNA fragment, encoding aa 1038-1175 of XRHAMM, was generated by PCR using the *Xenopus* cDNA library (Joukov, et al., 2001) as a template. The amplified fragment was subcloned into a pET29a(+) vector. The XRHAMM-CR³ cDNA fragment, encoding a mutant peptide, in which leucines 1106, 1113, and 1120 were replaced by arginines, was generated by mutagenesis of XRHAMM-Cwt using the QuickChange site-directed mutagenesis kit (Stratagene). XRHAMM-Cwt and XRHAMM-CR³ were expressed and purified as described for the Ran proteins. Recombinant human 6-His-cyclin A was produced and purified as described (Wohlschlegel, et al., 2001).

Rabbit polyclonal antibodies against the following proteins were used: XBRCA1 and XBARD1 (Joukov, et al., 2001); xMCM7 (Walter and Newport, 2000); xCAP-E (Smc2) (Hirano and Mitchison, 1994); *Xenopus* Topoisomerase II (Hirano and Mitchison, 1993); RCC1 (Dasso, et al., 1992); XRHAMM, xTPX2, and xNuMa (Groen, et al., 2004); hNuMA (a gift from D. Compton); histone H2B, phospho-histone H3 (Ser10), and hBRCA1 (Upstate); γ -tubulin Cy3 conjugate (Sigma-Aldrich). Mouse monoclonal antibodies against hBRCA1 (D9) (Santa Cruz) and α -tubulin, clone DM1A (Sigma), were used. The following labeled reagents, where appropriate, were used for immunodetection: goat anti-rabbit HRP-, FITC-, and rhodamine-conjugated IgG; goat anti-mouse FITC- and rhodamine-conjugated IgG; rabbit anti-mouse HRP-conjugated IgG (all from Jackson ImmunoResearch Laboratories); Protein A-HRP (Amersham Biosciences); and an S-Protein-HRP Conjugate (Novagen). The XBRCA1 antibodies were FITC conjugated using the EZ-LabelTM labeling kit (Pierce).

***Xenopus* egg extracts**

Three types of *Xenopus* egg extracts were used in this study: interphase-arrested extracts, metaphase-arrested extracts, and cycling extracts (Blow, 1993; Murray, 1991). After demembranated sperm chromatin is added to any of these extracts, it undergoes transformations that are determined by the cell cycle stage of the extract. Specifically, it becomes enclosed by a nuclear envelope and replicates in interphase-arrested extract. It also condenses and

participates in mitotic spindle formation in metaphase-arrested extract. In cycling egg extract, chromatin undergoes cyclical transitions between interphase and the mitotic state.

Extract naturally arrested in metaphase of meiosis II (CSF-arrested extract) was prepared by crushing unfertilized eggs in the presence of EGTA, which sequesters Ca^{2+} and thereby prevents destruction of CSF and cyclin B (Murray, 1991). Naïve sperm chromatin, when added to a CSF-arrested extract, enters the metaphase state, i.e. it condenses and induces formation of metaphase spindles. The arrest can be released by supplementing the extract with Ca^{2+} , which causes degradation of mitotic cyclins and entry of the extract into interphase.

For immunodepletions of extracts, Recombinant Protein A SepharoseTM beads (Amersham Biosciences) were incubated with affinity purified, rabbit XBARD1- and XBRCA1-specific antibodies (1 $\mu\text{g}/\mu\text{l}$ beads and 3 $\mu\text{g}/\mu\text{l}$ beads respectively) or XBARD1 and XBRCA1 antisera (1 $\mu\text{l}/\mu\text{l}$ beads and 3 $\mu\text{l}/\mu\text{l}$ beads respectively). The beads were washed twice with TBS (20 mM tris-HCl (pH 7.5), 150 mM KCl, 2.5 mM MgCl_2), and 5 times with ELB. Two to three rounds of immunodepletion, 45-60 min each, were carried out at 4°C. A bead-to-extract volume ratio of 1:5 was used for each round of depletion. Histone H1 kinase assays were carried out as described (Murray, 1991).

The replicated chromatin was cycled into mitosis by the addition of 75%-100% volume of the corresponding CSF-arrested extract or (in some experiments) 1 μM recombinant human cyclin A.

Analysis of chromatin in egg extracts

Sperm chromatin was added to extracts at concentrations 200-500 sperm nuclei/ μ l (experiments involving cycling egg extracts) or 1,000-4,000 sperm nuclei/ μ l (experiments involving interphase and CSF-arrested egg extracts). Chromatin morphology was analyzed by mixing 1 μ l of extract with an equal amount of Hoechst 33258 dye-containing fixing solution (Desai, et al., 1999) followed by IF microscopy.

For chromatin isolation assays, sperm chromatin-containing egg extract was diluted 10 fold with ice-cold ELB/0.2% Triton X100, layered onto 170 μ l of ELB/500 mM sucrose in 5x44 mm microcentrifuge tubes (Beckman), and centrifuged at 16,000g for 5 min at 4°C in a horizontal rotor. The supernatant was aspirated, and the pellet was washed with 400 μ l of ice-cold ELB/0.2% Triton X100 followed by an additional centrifugation through a sucrose cushion. The supernatant was aspirated, and the pellet dissolved in Laemmli sample buffer.

Immunoprecipitations

For IPs, 20 μ l of extract was diluted ten-fold with ice-cold buffer B (20 mM HEPES, pH7.8; 120 mM KCl; 2.5 mM MgCl₂; 5 mM EDTA; 0.25 M sucrose; 0.1% Triton X100; 25 mM iodoacetamide; 25 mM N-ethylmaleimide; 100mM NaF, 20 mM β -glycerophosphate, 2mM sodium orthovanadate, and 1 tablet/50 ml of the Complete Protease Inhibitor Cocktail (Boehringer Mannheim)). The

lysate was supplemented with the relevant Ab (1 to 2 μ g) and incubated on ice for 1hr. 5 μ l of Protein A Sepharose beads (Amersham Biosciences) were added to each lysate, and incubation continued for 40 min at 4°C, with slow rotation. Beads were retrieved by centrifugation, washed three times with ice-cold buffer B, and the eluted proteins were analyzed by SDS-PAGE/W-blotting.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Postmitotic nuclear assembly defect in BRCA/BARD1-depleted extract upon release of the metaphase arrest

(A) Mock-treated or BRCA1/BARD1-depleted, CSF-arrested extracts were supplemented with sperm chromatin (2,000 sperm nuclei/ μ l), and chromatin morphology was analyzed at the indicated time points by light microscopy (Phase) or fluorescence microscopy (Hoechst). Sperm chromatin was incubated for 1 h in extracts in which metaphase arrest had been released 20 min prior to chromatin addition ('Interphase'). Alternatively, the mitotic arrest was released 60 min after the addition of sperm chromatin ('Mitosis→Interphase'). Aliquots were withdrawn just before the release of arrest ('Mitosis') as well as 60 min and 90 min thereafter ('Mitosis→Interphase' '60", and '90" respectively). (B) BRCA1/BARD1 depletion does not affect mitotic exit in egg extract. Sperm chromatin was incubated in mock-treated and

BRCA1/BARD1-depleted CSF-arrested extracts, and metaphase arrest was released by the addition of calcium (Ca^{2+} , 0 min). Aliquots of each extract were withdrawn during mitosis and interphase at the indicated time points and analyzed for histone H1 kinase activity. The phosphorylated products were analyzed by SDS-PAGE followed by autoradiography. (C and D) Rescue of postmitotic nuclear assembly defect with recombinant BRCA1/BARD1. Mock-treated and BRCA1/BARD1-depleted CSF-arrested extracts were released from metaphase arrest, supplemented with sperm chromatin (2,000 sperm nuclei/ μl) and ELB or rBRCA1/BARD1, and incubated at 22°C for 90 min. Mitosis was generated by the addition of 75% of the corresponding CSF-arrested extract, and after 60-min incubation, the extracts were driven into a second (postmitotic) interphase by the addition of 0.5 mM Ca^{2+} . The morphology of chromatin was analyzed 80 min after the addition of Ca^{2+} . Representative nuclei (C) and quantification of the nuclei diameter (mean \pm SEM) (D) are shown.

Figure S2. The recombinant *Xenopus* BRCA1/BARD1 heterodimers

(A) Analysis of recombinant *Xenopus* wt BRCA1/BARD1 and BRCA1(I26A)/BARD1 by SDS-PAGE and gel staining. SeeBlue® Plus2 protein molecular weight standards are shown on the left. (B) Analysis of ubiquitin E3 ligase activity of rBRCA1/BARD1 and rBRCA1(I26A)/BARD1 (autoubiquitination and ubiquitination of histone H2B). The reactions were carried out in the presence of ^{32}P -ubiquitin, as described for human

BRCA1/BARD1 (Mallery, et al., 2002). The ubiquitinated products were analyzed by SDS-PAGE followed by autoradiography. Mono-ubiquitinated histone H2B is indicated by an arrow.

Figure S3. SiRNA-mediated depletion of BRCA1/BARD1 in HeLa cells and spindle structures assembled in control and BRCA1/BARD1-deficient cells

(A) HeLa cells were transfected with a control siRNA or human BRCA1+BARD1 siRNAs. Cells were lysed and analyzed by W-blotting with the indicated antibodies. (B) Control- and BRCA1/BARD1-siRNA-treated HeLa cells were fixed in methanol/acetone and stained with an anti- α -tubulin Ab and DAPI. Representative mitotic figures in control-treated ('C') and BRCA1/BARD1-deficient ('Δ') cells are shown. Microtubules are in red, and chromosomes are pseudocolored green.

Figure S4. Chromatin-induced MT asters and spindles assembled in mock-treated and BRCA1/BARD1-depleted extracts (time course)

CSF-arrested extracts were supplemented with rhodamine-labeled tubulin, Alexa-Fluor-488-labeled TPX2 Ab, and sperm chromatin. Aliquots of extracts were withdrawn at the indicated times, fixed, and analysed by IF microscopy. Representative mitotic figures are shown for each time point. Note unfocused aster/spindle poles and failure of TPX2 to accumulate on centers of these poles in BRCA1/BARD1-depleted extract.

Figure S5. Schematic representation of functional communication between BRCA1/BARD1 and SAFs during Ran-dependent MT assembly

SUPPLEMENTAL TABLE

<div> Additions Depletions </div>	Buffer	α -XRHAMM (20 ng/ μ l)	XRHAMM-Cwt
Mock	1. Normal	2. Normal	3. Pole/TPX2 defect ++
Δ BRCA1	4. Pole/TPX2 defect ++	5. RESCUE of (4) +++	6. Pole/TPX2 defect ++++
Δ XRHAMM	7. Asters malformed ++	8. Asters malformed +++	9. RESCUE of (7) ++
Δ BRCA1+ Δ XRHAMM	10. RESCUE of (4) ++	N/A	N/A

Table S1. Effects of the XRHAMM Ab (α -XRHAMM) and XRHAMM-Cwt on the morphology of RanGTP-induced MT asters and accumulation of TPX2 on aster poles in the mock-treated extract (Mock), and in extracts depleted of BRCA1/BARD1 (Δ BRCA1), XRHAMM (Δ XRHAMM), or both BRCA1/BARD1 and XRHAMM (Δ BRCA1+ Δ XRHAMM). The degree of the effect corresponds to the number of '+', from (+), minimal effect; to (++++), maximal effect. N/A, effect non-assessed.

SUPPLEMENTAL REFERENCES

Blow, J. J. (1993). Preventing re-replication of DNA in a single cell cycle: evidence for a replication licensing factor. *J. Cell. Biol.* 122, 993-1002.

Dasso, M., Nishitani, H., Kornbluth, S., Nishimoto, T., and Newport, J. W. (1992). RCC1, a regulator of mitosis, is essential for DNA replication. *Mol. Cell. Biol.* 12, 3337-3345.

Dasso, M., Seki, T., Azuma, Y., Ohba, T., and Nishimoto, T. (1994). A mutant form of the Ran/TC4 protein disrupts nuclear function in *Xenopus laevis* egg

extracts by inhibiting the RCC1 protein, a regulator of chromosome condensation. *EMBO J.* **13**, 5732-5744.

Desai, A., Murray, A., Mitchison, T. J., and Walczak, C. E. (1999). The use of *Xenopus* egg extracts to study mitotic spindle assembly and function in vitro. *Methods Cell Biol.* **61**, 385-412.

Groen, A. C., Cameron, L. A., Coughlin, M., Miyamoto, D. T., Mitchison, T. J., and Ohi, R. (2004). XRHAMM functions in ran-dependent microtubule nucleation and pole formation during anastral spindle assembly. *Curr. Biol.* **14**, 1801-1811.

Hirano, T., and Mitchison, T. J. (1993). Topoisomerase II does not play a scaffolding role in the organization of mitotic chromosomes assembled in *Xenopus* egg extracts. *J. Cell Biol.* **120**, 601-612.

Hirano, T., and Mitchison, T. J. (1994). A heterodimeric coiled-coil protein required for mitotic chromosome condensation *in vitro*. *Cell* **79**, 449-458.

Ikura, T., Ogryzko, V. V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000). Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell* **102**, 463-473.

Joukov, V., Chen, J., Fox, E. A., Green, J. B., and Livingston, D. M. (2001). Functional communication between endogenous BRCA1 and its partner, BARD1, during *Xenopus laevis* development. *Proc. Natl. Acad. Sci. USA* **98**, 12078-12083.

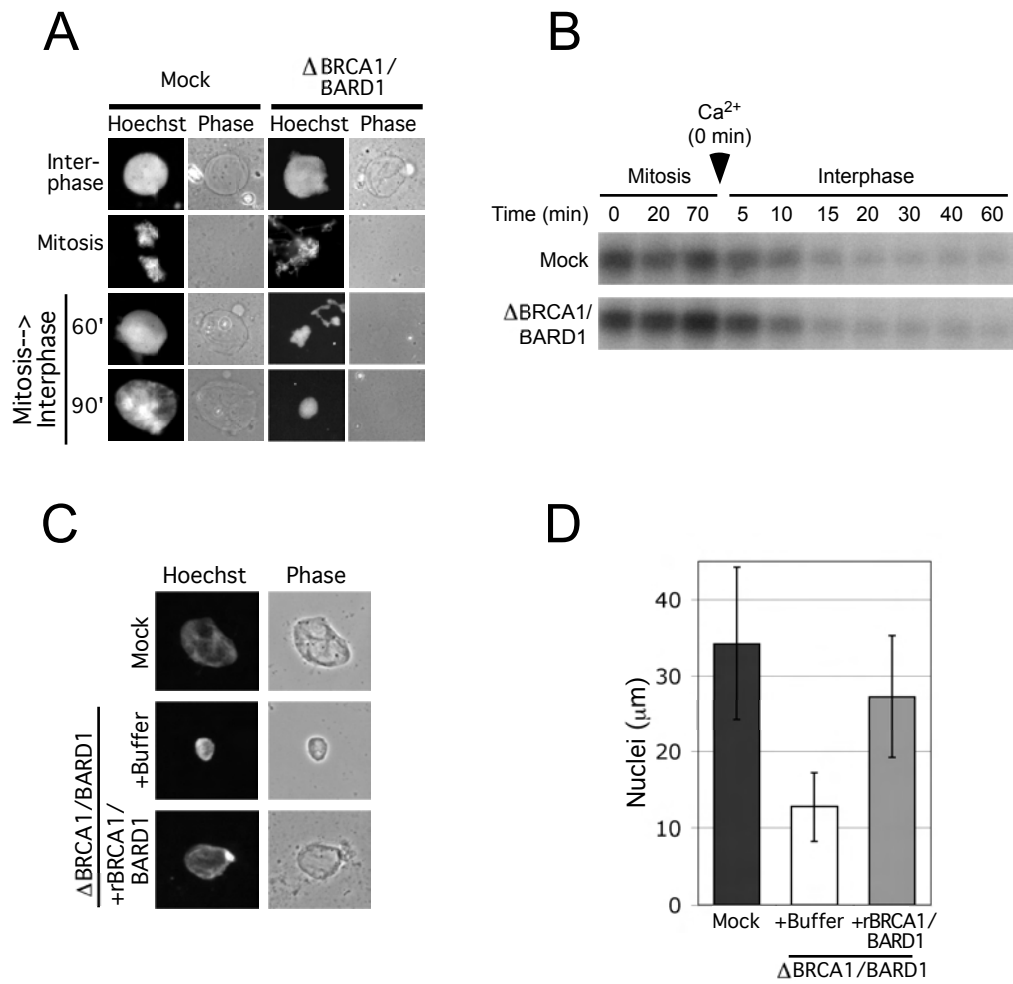
Mallery, D. L., Vandenberg, C. J., and Hiom, K. (2002). Activation of the E3 ligase function of the BRCA1/BARD1 complex by polyubiquitin chains. *EMBO J.* **21**, 6755-6762.

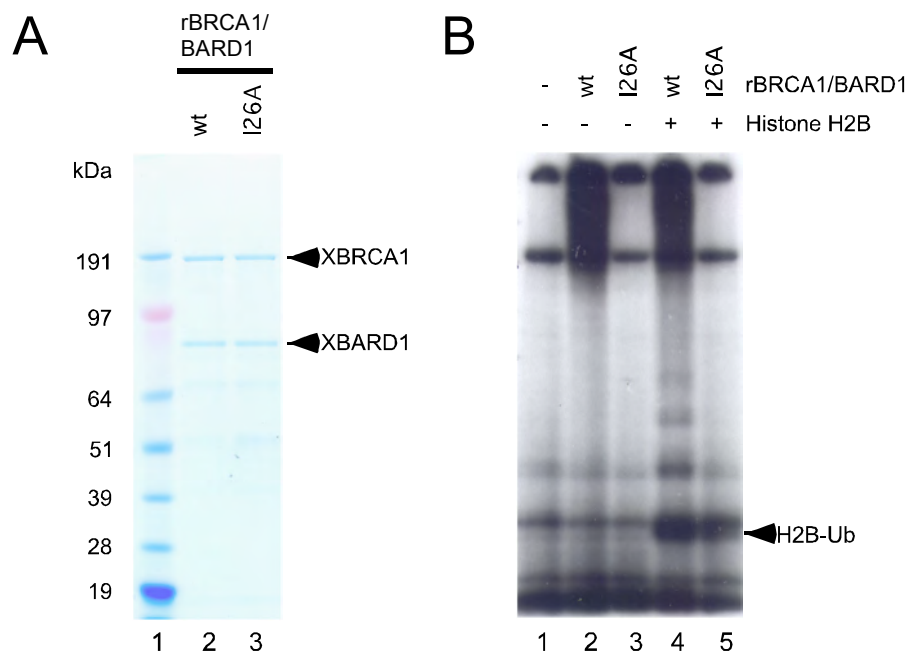
Minshull, J., Sun, H., Tonks, N. K., and Murray, A. W. (1994). A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. *Cell* **79**, 475-486.

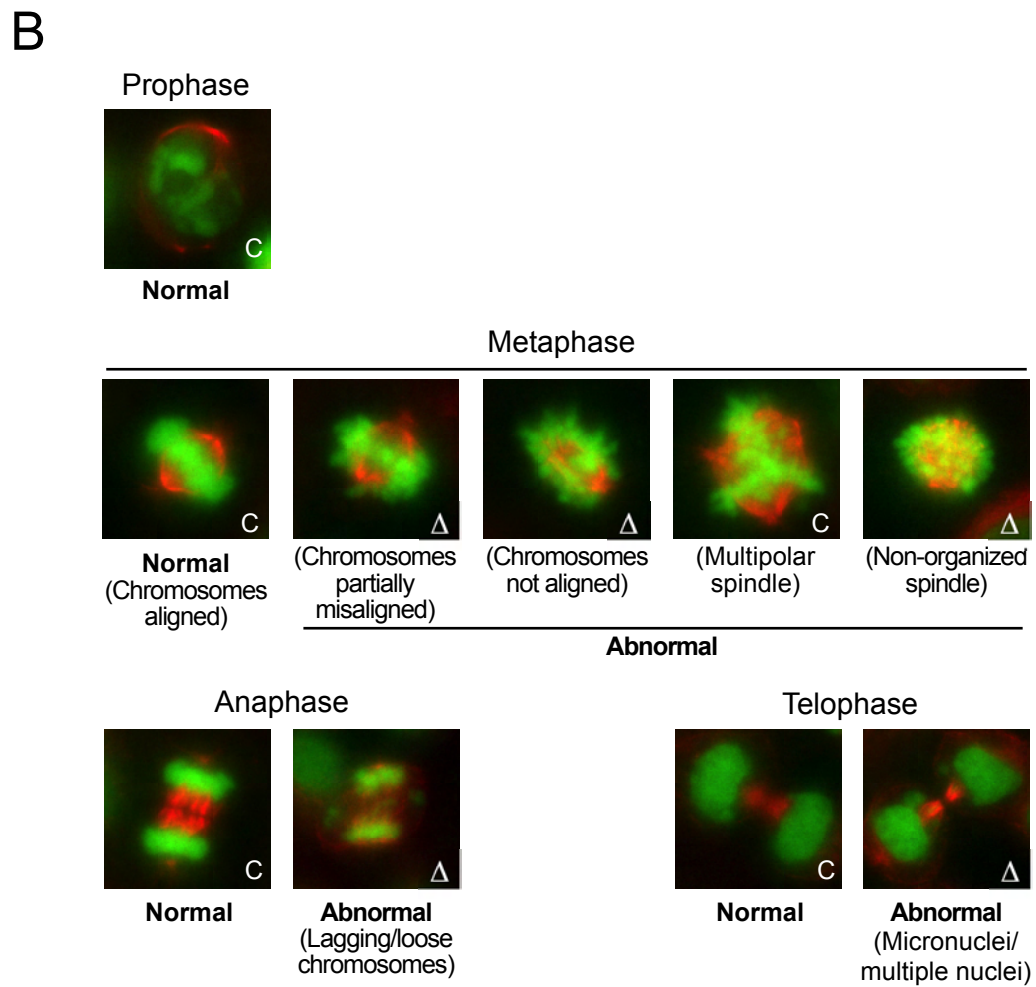
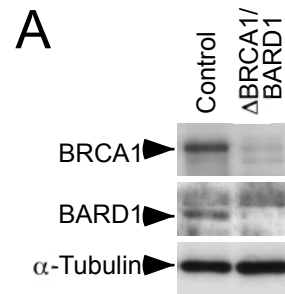
Murray, A. W. (1991). Cell cycle extracts. *Methods Cell Biol.* **36**, 581-605.

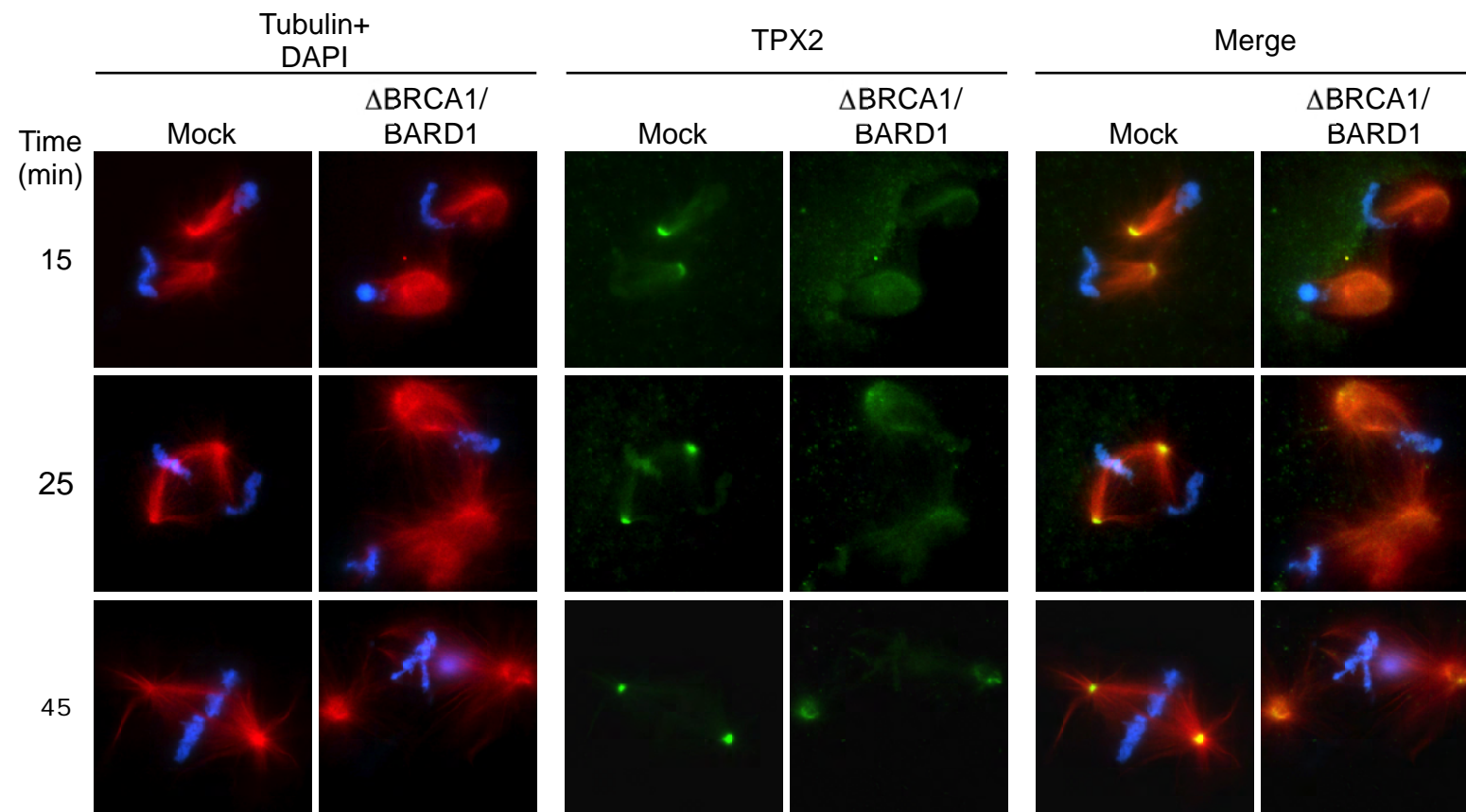
Walter, J., and Newport, J. (2000). Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. *Mol. Cell* **5**, 617-627.

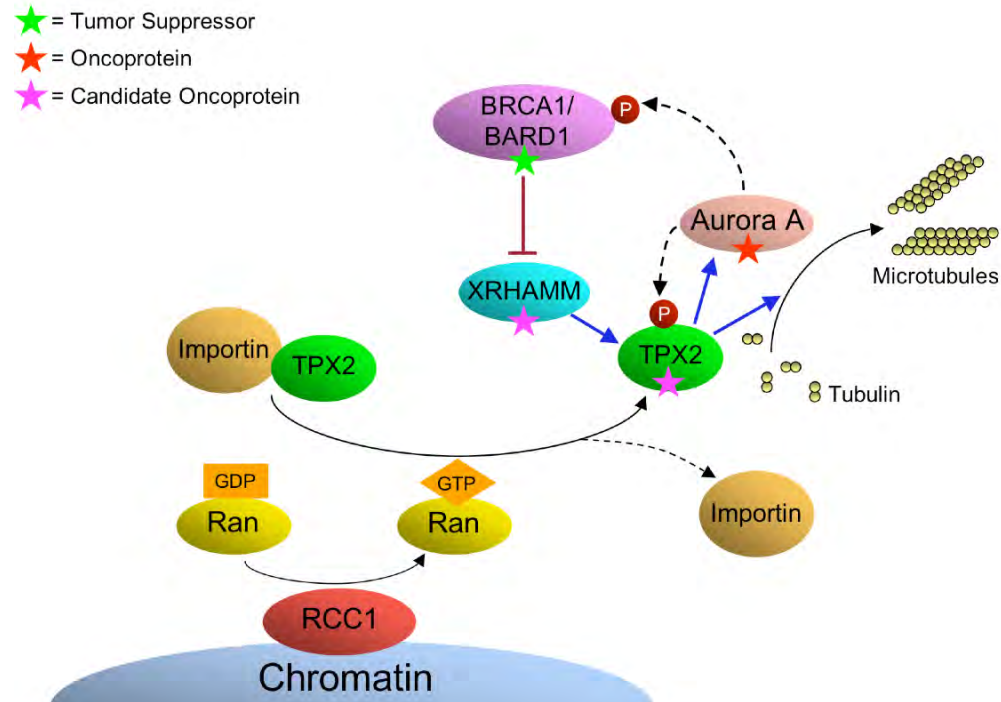
Wohlschlegel, J. A., Dwyer, B. T., Takeda, D. Y., and Dutta, A. (2001). Mutational analysis of the Cy motif from p21 reveals sequence degeneracy and specificity for different cyclin-dependent kinases. *Mol. Cell. Biol.* **21**, 4868-4874.











Joukov et al., Figure S5

BRCA1/BARD1- MEDIATED ASSOCIATION OF RCC1 WITH CHROMATIN IS REQUIRED FOR THE FIDELITY OF MITOSIS AND MITOTIC EXIT

Vladimir Joukov¹, Tanya Prokhorova², Ruth Gerson¹, Erinn White¹, Johannes C. Walter² and David M. Livingston¹

¹The Dana-Farber Cancer Institute and the ²Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115, USA

Germ line mutations in the BRCA1 gene predispose to breast and/or ovarian cancer. BRCA1 dimerizes with a structurally related protein BARD1, and the heterodimers possess an E3 ligase activity of unknown *in vivo* specificity and function. Both BRCA1 and BARD1 are required for cell proliferation and chromosome stability, but the molecular basis of this requirement is unknown. To elucidate the role of BRCA1/BARD1 in cell proliferation, we employ a cell-free system derived from *Xenopus* egg extracts, which oscillates between S phase and mitosis *in vitro* ("cycling extract") and faithfully recapitulates many aspects of the cell cycle and its regulation. Extracts depleted of BRCA1/BARD1 with specific antibodies exhibit defects in mitosis and the mitosis to interphase transition. In mitosis, the mitotic spindles appear disorganized, and chromosomes fail to congress at the metaphase plate. Although depleted extract exits mitosis, it is unable to undergo the following S phase. The defective S phase can be explained by the observation that in the absence of BRCA1/BARD1, the nuclear envelope is not formed properly, thereby resulting in DNA replication initiation failure. Indeed, in the absence of BRCA1/BARD1, the replication initiation factor Cdc45, whose binding to the pre-replication complex requires an intact nuclear envelope, does not load properly onto postmitotic chromatin, the latter fails to decondense, and Ser10 of histone H3 does not undergo dephosphorylation.

The defects seen in BRCA1/BARD1 depleted extracts were accompanied by deficient chromatin binding of the small GTPase Ran nucleotide exchange factor, RCC1, to chromatin. The latter observation along with the fact that formation of both the mitotic spindle and the nuclear envelope requires generation of RanGTP by the chromatin-bound RCC1, suggest that dysregulation of the Ran pathway is material to the former defects. Supporting this notion, RCC1 chromatin loading and other defects were rescued by supplementing the immunodepleted extract with either recombinant BRCA1/BARD1 heterodimers or Ran-GTP. In addition, ablation of BRCA1/BARD1 by RNAi depleted HeLa cell chromosomes of RCC1 and led to gross mitotic abnormalities.

Taken together, our results reveal a hitherto unrecognized role of BRCA1/BARD1 in regulating the Ran pathway and point to a mechanism that might underlie the known function of BRCA1 in the control of cell proliferation, chromosome stability and tumor suppression.

P51-15: BRCA1/BARD1 MEDIATED TARGETING OF RCC1 TO CHROMATIN IS REQUIRED FOR THE FIDELITY OF MITOSIS AND MITOTIC EXIT

Vladimir Joukov,¹ Tatyana Prokhorova,² Ruth Gerson,¹ Erinn White,¹ Johannes C. Walter,² and David M. Livingston¹

¹Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA; ²Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA

E-mail: vladimir_joukov@dfci.harvard.edu

Germ line mutations in BRCA1 predispose to breast and/or ovarian cancer with high penetrance and, in the case of breast cancer, early onset. BRCA1 is a nuclear protein that heterodimerizes with BARD1, a BRCA1 structural relative suspected of playing a tumor suppressing role in its own right. Both BRCA1 and BARD1 are required for cell proliferation and chromosome stability, but the molecular basis for this requirement is poorly understood. To elucidate biochemical function of BRCA1/BARD1, we employ *Xenopus* egg extract, a well-established, highly tractable cell free system that recapitulates many key intracellular processes and allows dissection of their molecular mechanisms. Such approach provides an opportunity to bypass the cell survival problem that has been hampering BRCA1 studies. Because of these unique abilities, egg extracts have previously played an instrumental role in studying of many proteins involved in cancer and other diseases. Now they can be used to study BRCA1.

Through the analysis of *Xenopus* egg extract, the BRCA1/BARD1 heterodimers can be shown, for the first time, to regulate mitosis and the mitosis to interphase transition by targeting the small GTPase, Ran, nucleotide exchange factor, RCC1, to chromatin. Depleting egg extract of BRCA1/BARD1 heterodimers inhibited RCC1 binding to mitotic chromosomes, disrupted the mitotic spindle and nuclear envelope assembly and prevented DNA replication initiation in postmitotic S phase. These defects were rescued by supplementing depleted extract with either recombinant BRCA1/BARD1 heterodimers or Ran-GTP. Similarly, depletion of BRCA1/BARD1 by RNA interference in HeLa cells depleted RCC1 from metaphase chromosomes and led to the defects in mitotic spindle assembly and anaphase chromosome segregation.

We propose that regulation of the Ran pathway is an essential, cell cycle-associated function of BRCA/BARD1 that contributes to their multifaceted role in cell proliferation, maintenance of chromosome stability and tumor suppression. Further analysis of the connection between BRCA1/BARD1 and the Ran pathway might help to uncover mechanisms that allow breast cancer cells to survive and proliferate without BRCA1. Such knowledge, in turn, could spur development of novel methods of cancer chemotherapy and chemoprevention.

The U.S. Army Medical Research and Materiel Command under W81XWH-04-1-0524 supported this work.

The Era of Hope Department of Defense Breast Cancer Research Program Meeting (Philadelphia, Pennsylvania, USA); June 8-11, 2005.

BRCA1/BARD1 SUPPORTS THE RAN PATHWAY IN ITS CONTROL OF MITOTIC FIDELITY

Vladimir Joukov¹, Aaron C. Groen³, Tanya Prokhorova², Ruth Gerson¹, Erinn White¹, Alison Rodriguez¹, Johannes C. Walter², and David M. Livingston¹

¹Dana-Farber Cancer Institute and Harvard Medical School,

²Department of Biological Chemistry and Molecular Pharmacology, and

³Department of Systems Biology, Harvard Medical School, Boston, Massachusetts 02115, USA

Germ line mutations in the BRCA1 gene predispose to breast and ovarian cancer. BRCA1 dimerizes with a structurally related protein, BARD1, and the resulting heterodimer exhibits an E3 ubiquitin ligase activity of unknown *in vivo* specificity and function. Both BRCA1 and BARD1 participate in cell proliferation and chromosome stability control, the molecular basis for which is poorly understood. Although BRCA1 has been implicated in a variety of cellular processes, the most commonly accepted view is that it controls genome integrity by regulating DNA damage responses.

To elucidate the molecular function of BRCA1/BARD1, we employ *Xenopus* egg extracts, a well-established, biochemically tractable, cell-free system that recapitulates S phase and mitosis. In these extracts, BRCA1/BARD1 was found to be critical for normal execution of mitosis and mitosis-to-interphase transition, but not for S phase progression. Our observations implicate BRCA1/BARD1 in the regulation of chromatin-driven microtubule/mitotic spindle organization independently of centrosomes. This function operates downstream of RanGTPase and depends upon BRCA1/BARD1 E3 ubiquitin ligase activity. BRCA1/BARD1-depleted extracts exhibited defects in chromosome congression and spindle pole assembly, and failure of TPX2, spindle organizer and Ran target, to concentrate on spindle poles. BRCA1/BARD1 was also found to associate with the spindle assembly factors, NuMA and XRHAMM. Furthermore, BRCA1/BARD1-depleted extracts also revealed a defect in the association of RCC1, the nucleotide exchange factor for Ran, with metaphase chromatin that resulted from improper spindle assembly and led to mitotic exit abnormalities. Both BRCA1/BARD1-depleted extracts and mammalian cells exhibited these abnormal phenotypes.

These data implicate BRCA1/BARD1 and its E3 ubiquitin ligase activity in the regulation of Ran-dependent mitotic microtubule organization and suggest that the latter function underlies, at least in part, the role of BRCA1/BARD1 in cell proliferation and chromosome stability control.

Cold Spring Harbor Laboratory Meeting - The Cell Cycle (Cold Spring Harbor, New York, USA); May 17-21, 2006.

BRCA1/BARD1 CONTROLS CHROMATIN-DRIVEN MITOTIC SPINDLE ASSEMBLY

Vladimir Joukov¹, Aaron C. Groen³, Tanya Prokhorova², Ruth Gerson¹, Erinn White¹, Alison Rodriguez¹, Johannes C. Walter², and David M. Livingston¹

¹Dana-Farber Cancer Institute and Harvard Medical School, ²Department of Biological Chemistry and Molecular Pharmacology, and ³Department of Systems Biology, Harvard Medical School, Boston, Massachusetts 02115, USA

The breast and ovarian tumor suppressor BRCA1 heterodimerizes with a structurally related protein, BARD1, and the resulting heterodimer exhibits an E3 ubiquitin ligase activity of unknown *in vivo* specificity and function. BRCA1- and BARD1-deficient cells and embryos exhibit proliferation deficiency and aneuploidy, which are often accompanied by centrosome amplification. The molecular basis for these defects is poorly understood. Although BRCA1 has been implicated in a variety of cellular processes, the most commonly accepted view is that it controls genome integrity by regulating DNA damage responses.

We have examined the function of BRCA1/BARD1 heterodimer using *Xenopus* egg extracts, a biochemically tractable, cell-free system that recapitulates S phase and mitosis/meiosis, and allows to bypass the problem of non-viability of BRCA1- and BARD1-deficient cells. In these extracts, BRCA1/BARD1 was found to control mitotic fidelity by regulating chromatin-driven mitotic spindle assembly independently of centrosomes. This function operates downstream of RanGTPase and depends upon BRCA1/BARD1 E3 ubiquitin ligase activity. BRCA1/BARD1-depleted extracts exhibited defects in nuclear envelope and spindle pole assembly, in chromosome congression at the metaphase plate, and failure of TPX2, the major spindle organizer and Ran target, to concentrate on spindle poles. The mitotic spindle defects were rescued either by supplementing extracts with recombinant BRCA1/BARD1 or, unexpectedly, by immunodepletion or antibody-mediated inhibition of XRHAMM, a recently emerged TPX2 partner. Moreover, in meiotic extract, the endogenous BRCA1/BARD1 formed complexes with XRHAMM, TPX2, and NuMa, another spindle organizer and Ran target. Abnormalities similar to those seen in egg extracts were also detected in BRCA1/BARD1-deficient cultured mammalian cells.

These observations reveal a heretofore-unrecognized E3 ligase-driven mitotic function of BRCA1/BARD1 that contributes to its role in chromosome stability control. Moreover, by implicating BRCA1/BARD1 in the regulation of key participants in the Ran-dependent mitotic spindle assembly, which are frequently deregulated in cancer cells (i.e. RHAMM, TPX2, and Aurora A), the study suggests a novel pathway for BRCA1/BARD1-mediated tumor suppression.

Cold Spring Harbor Laboratory Meeting - Mechanisms and Models of Cancer (Cold Spring Harbor, New York, USA); August 16-20, 2006 (Oral presentation).

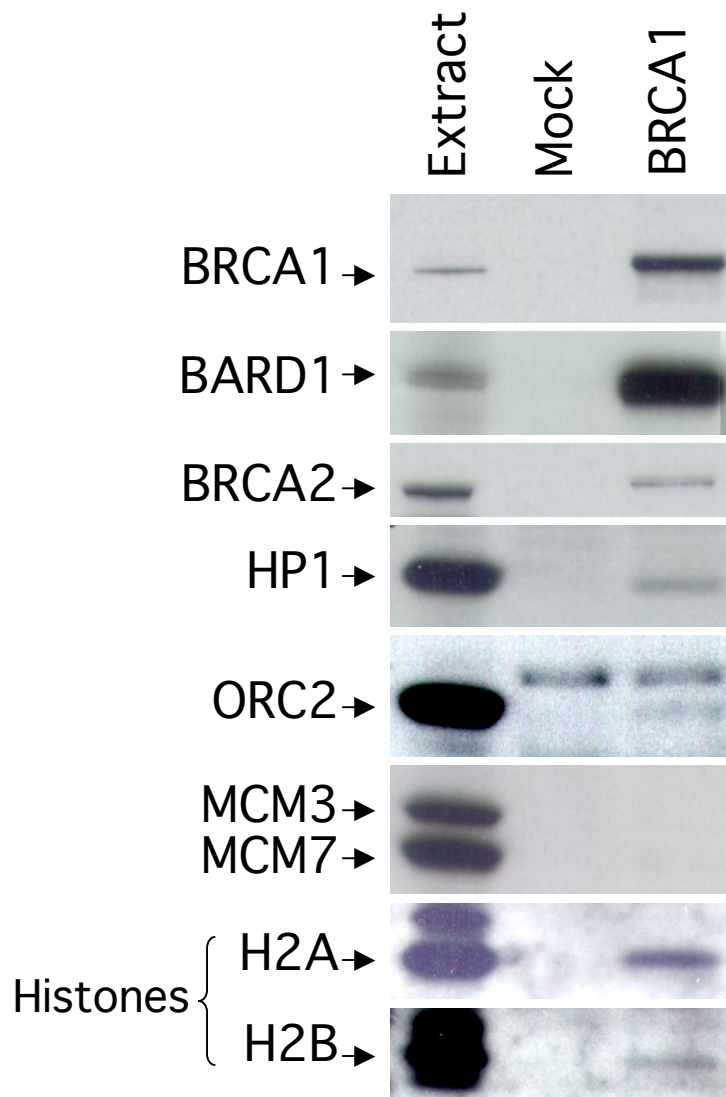


Figure 1. Western blot analysis of endogenous BRCA1/BARD1 complexes isolated from interphase egg extract. A rabbit polyclonal antibody directed against a peptide encompassing amino acids 164-179 of *Xenopus* BRCA1 (EVTRLIPCRQKKPKKE) or non-immune rabbit IgG (Mock) were cross-linked to Sepharose beads and the resulting immunoaffinity matrix was incubated with interphase egg extract at +4°C for 1 hr with gentle rotation. The beads were isolated and washed four times with ELB buffer containing 0.05% Tween 20 and protease inhibitors. The proteins were eluted using the aforementioned peptide and analyzed by Western blotting with the indicated antibodies.

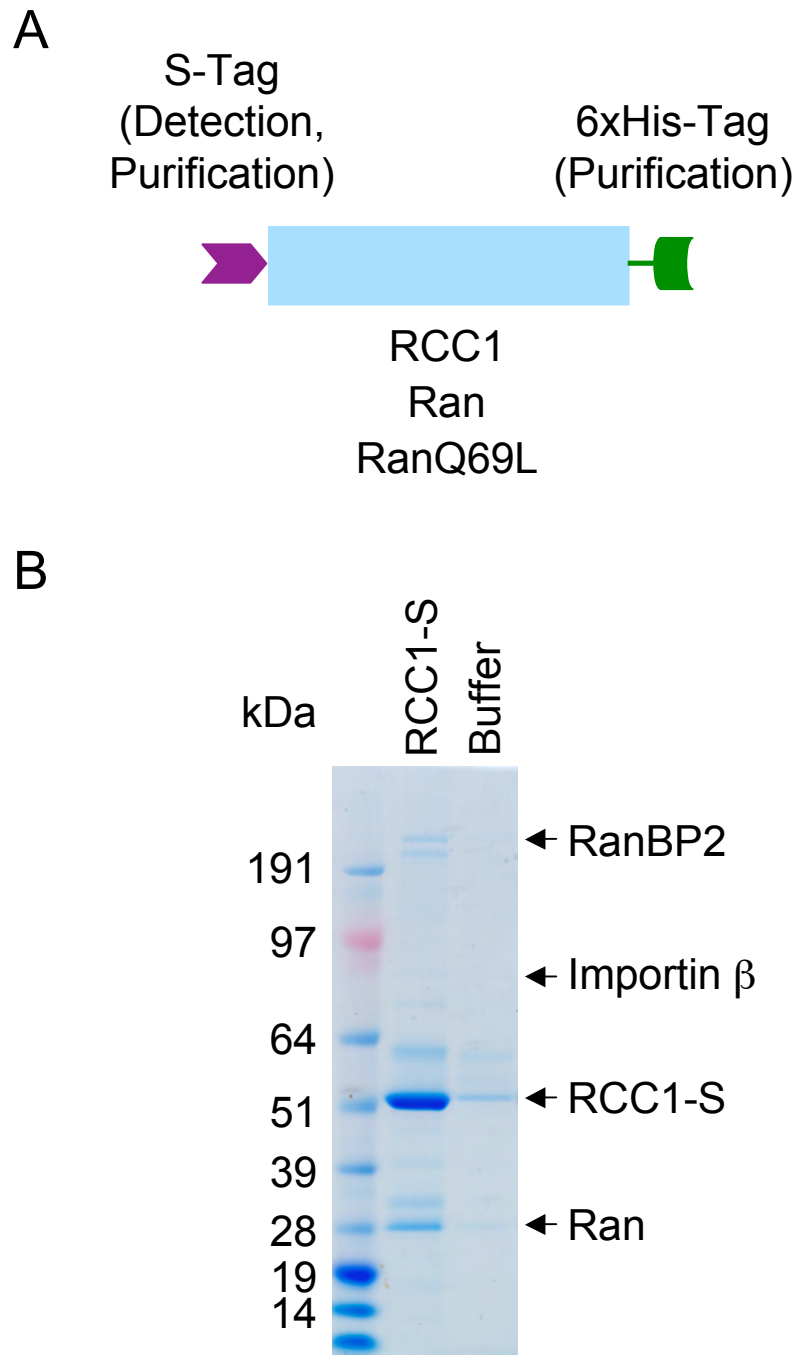


Figure 2. The recombinant RCC1 binds Ran and Ran-binding proteins in egg extract

(A) A schematic diagram of the recombinant proteins of the Ran pathway that were used in the study. RCC1, wtRan, and RanQ69L bearing an N-terminally located S-tag and a C-terminally located 6xHis tag were produced in bacteria and purified using the Ni-NTA resin. (B) RCC1-S binds Ran and Ran-interacting proteins in egg extract. Interphase egg extract was supplemented with ELB (buffer) or S-tagged RCC1 (RCC1-S) and incubated for 30 min at 22°C. RCC1-S was isolated using S Protein Agarose (Novagen) and analyzed by SDS-PAGE and QuickBlue™ staining. SeeBlue® Plus2 protein molecular weight standards (5 μ l) are shown on the left.

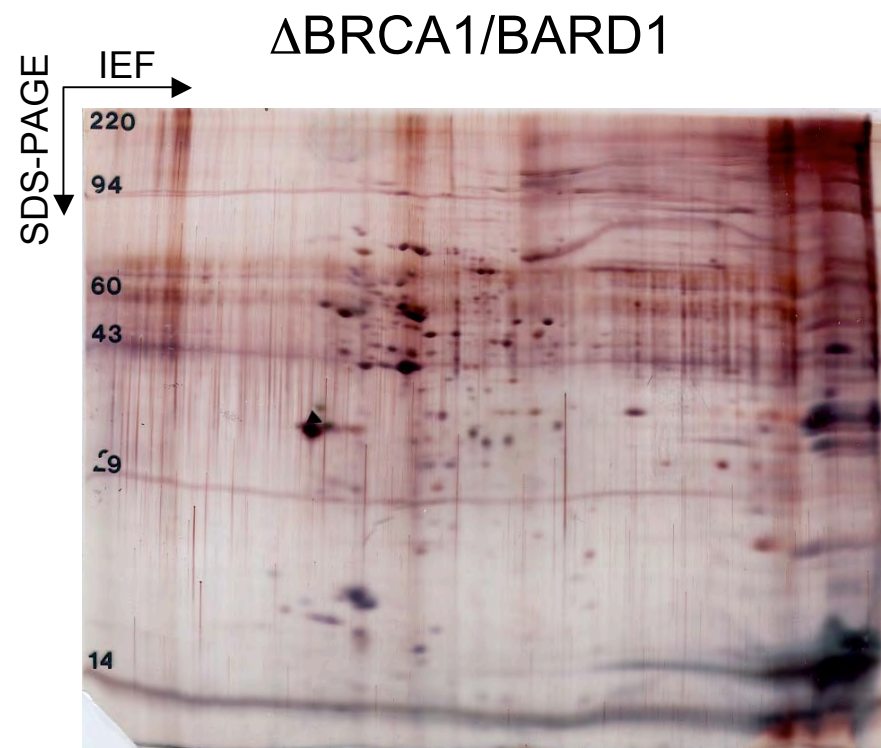
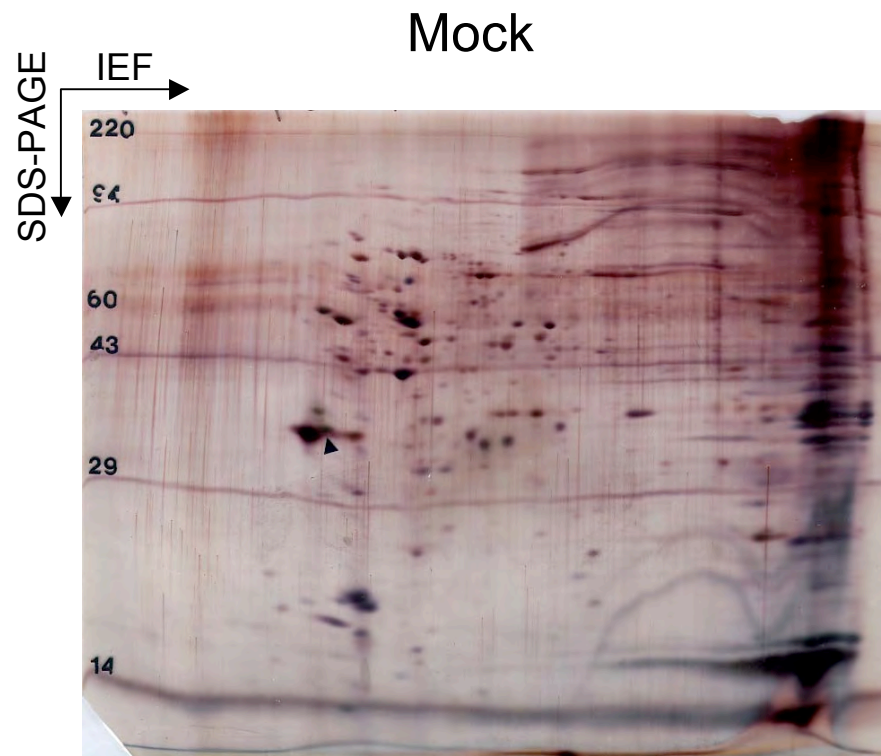


Figure 3. Two-dimensional gel electrophoresis of chromatin-bound proteins isolated from the mock-treated (Mock) and BRCA1/BARD1-depleted (Δ BRCA1/BARD1) interphase egg extracts.

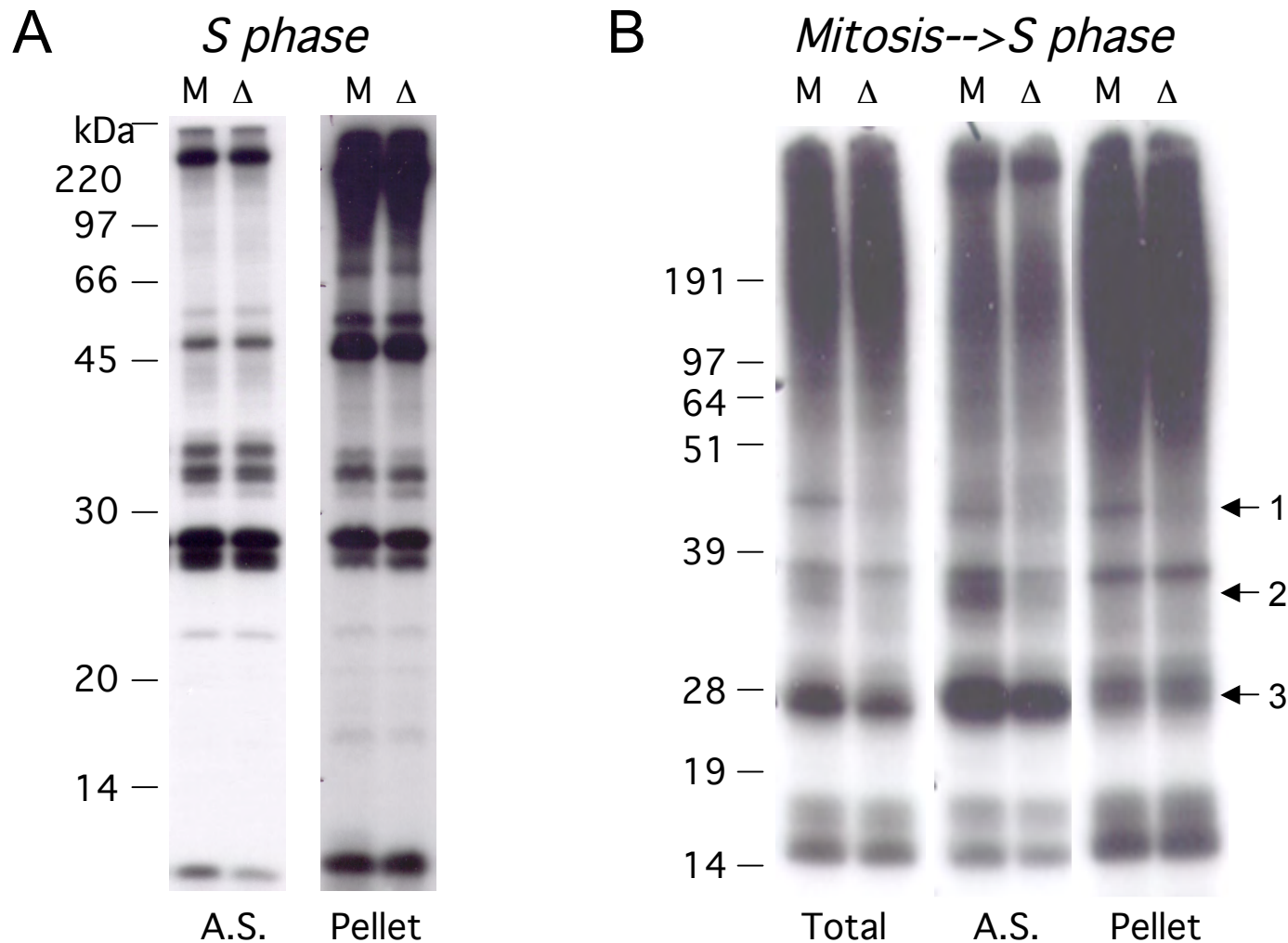


Figure 4. The effect of BRCA1/BARD1 depletion on the ubiquitination of proteins associated with the interphase (A) or postmitotic (B) chromatin

(A) Sperm chromatin was incubated for 1 hr in the mock-treated (M) or BRCA1-depleted (Δ) interphase egg extracts supplemented with ^{32}P -ubiquitin. Chromatin was isolated and subjected to the extraction with 0.5 M HCl. Acid soluble (A.S.) and acid non-soluble (Pellet) fractions were analyzed by electrophoresis followed by autoradiography.

(B) Sperm chromatin was incubated for 1 hr in metaphase-arrested extracts supplemented with ^{32}P -ubiquitin. The arrest was released by the addition of 0.5 mM Ca^{2+} and after 1 hr, chromatin was isolated and analyzed as in (A). Positions of three products whose ubiquitination is decreased in BRCA1/BARD1-depleted extract are indicated by arrows.

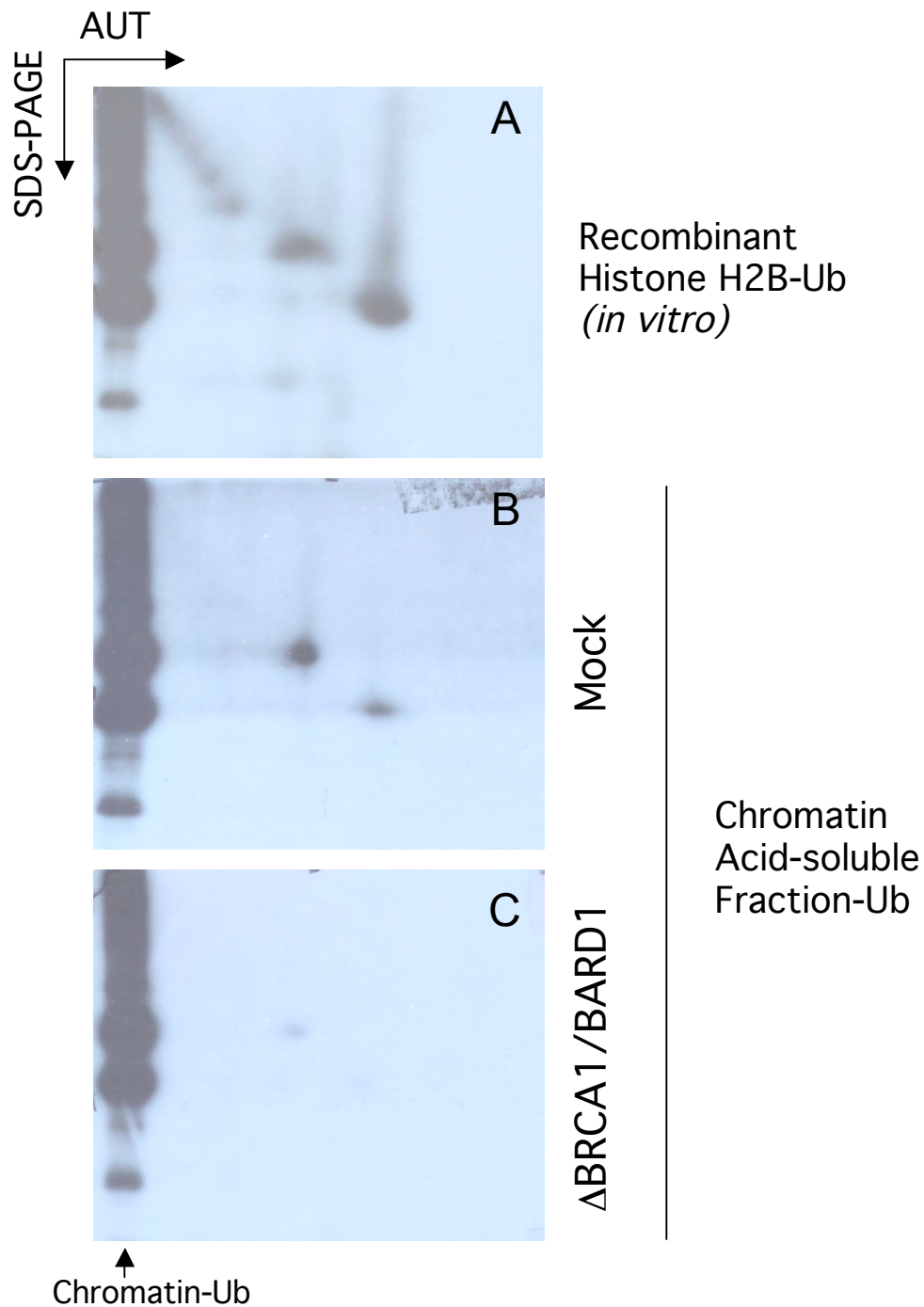


Figure 5. Two-dimensional gel electrophoresis of the *in vitro* ubiquitinated recombinant histone H2B (A) and the acid-soluble fractions of postmitotic chromatin isolated from the mock-treated (B) and BRCA1/BARD1-depleted (C) extracts. First dimension: acetic acid-urea-Triton X-100 (AUT) electrophoresis (Dimitrov and Wolffe, 1997). Second dimension: SDS-PAGE.

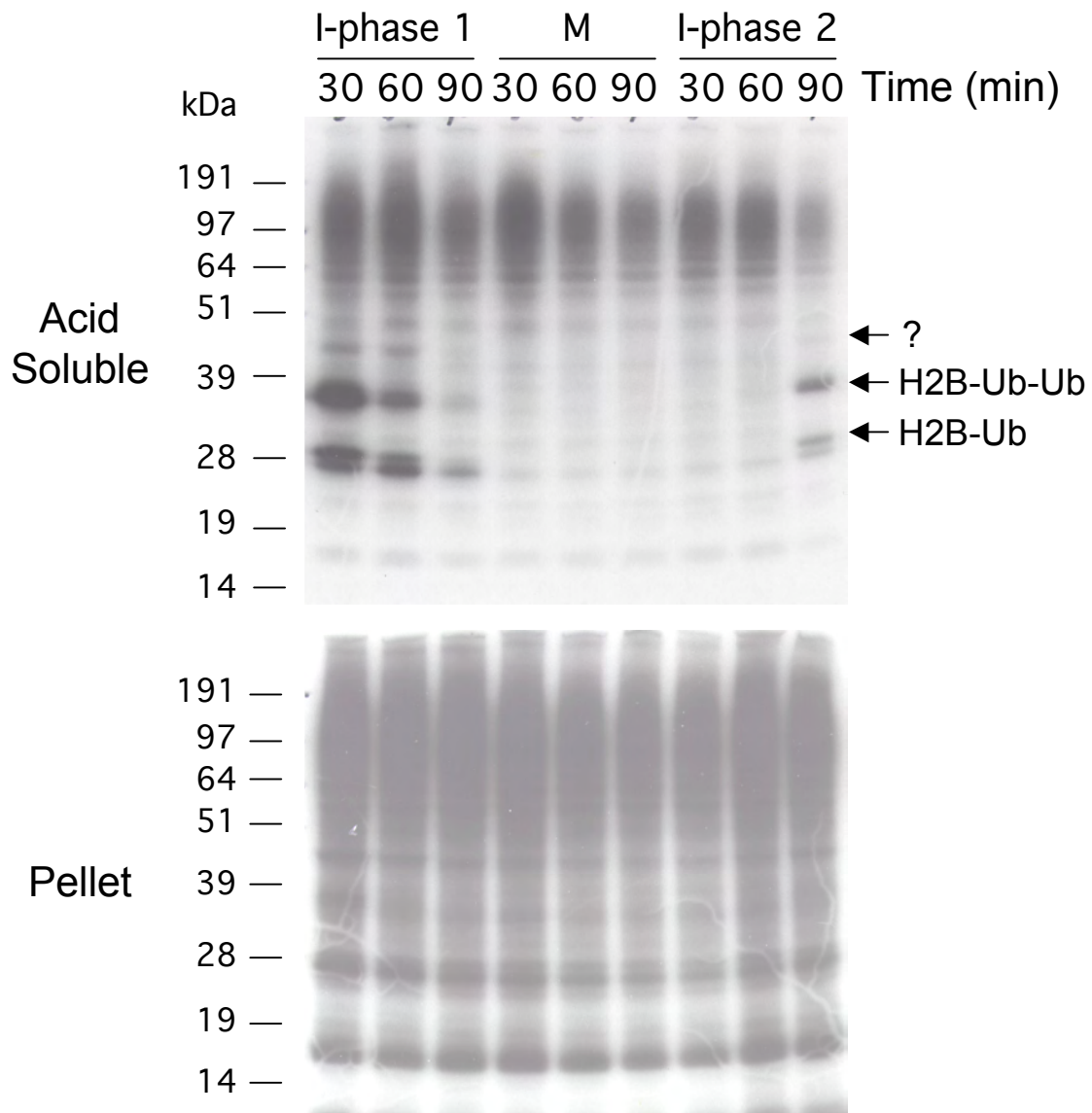
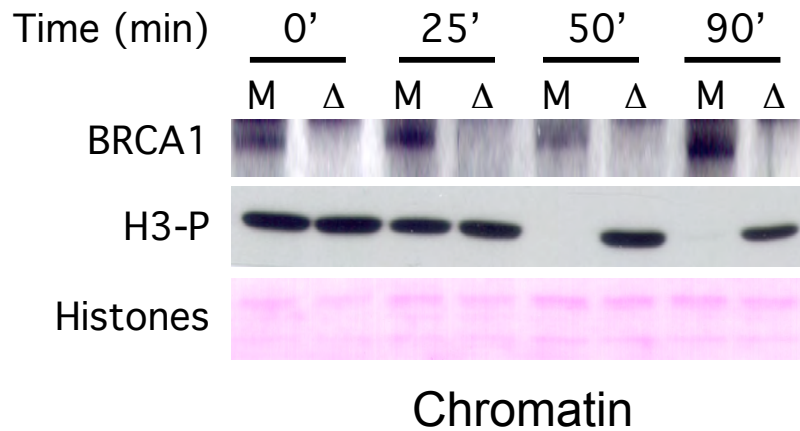


Figure 6. Interphase-specific ubiquitination of histone H2B (arrows) in acid-soluble chromatin fraction. Sperm chromatin was incubated for the indicated time in the interphase-arrested (I-phase 1) or in the metaphase-arrested (M) egg extracts supplemented with ^{32}P -ubiquitin. Alternatively, sperm chromatin was pre-incubated for 1 hr in metaphase-arrested extract supplemented with ^{32}P -ubiquitin followed by the addition of Ca^{2+} to release the arrest. Chromatin was isolated at the indicated times after the addition of Ca^{2+} (I-phase2). Acid-soluble (upper panel) and acid-non-soluble (lower panel) proteins were analyzed by SDS-PAGE. Two ubiquitinated protein products were identified as mono- and di-ubiquitinated forms of histone H2B (See Figure 5).

A



B

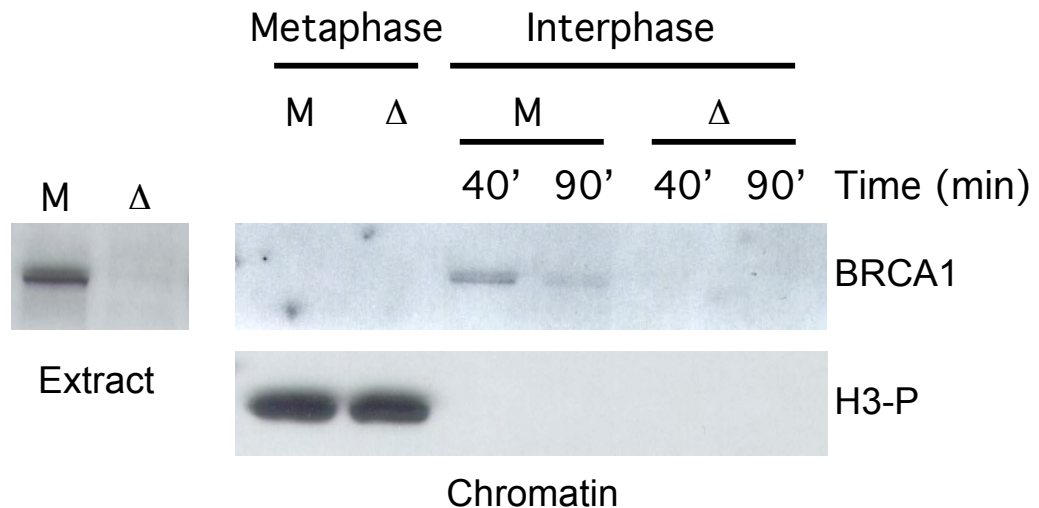


Figure 7. BRCA1/BARD1 is required for postmitotic chromatin decondensation in low-speed egg extract

(A) Sperm chromatin (2,000 sperm/ μ l) was incubated at 22°C for 60 min in the mock-treated (M) or the BRCA1/BARD1-depleted (Δ) metaphase-arrested *low-speed* extract followed by the release of the arrest. Chromatin was isolated just before the release of arrest (0') as well as 25, 50 min, and 90 min thereafter, and analysed by Western blotting using antibodies directed against xBRCA1 and Ser10-phosphorylated histone H3. Histones were detected by staining the membrane with Ponceau-S solution. (B) Sperm chromatin was incubated in the *high-speed* metaphase-arrested mock-treated or BRCA1/BARD1-depleted egg extracts and isolated either prior to or after the release of arrest at the indicated times. Chromatin was analyzed as in (A). Left panel, extracts were probed with the xBRCA1 antibody.

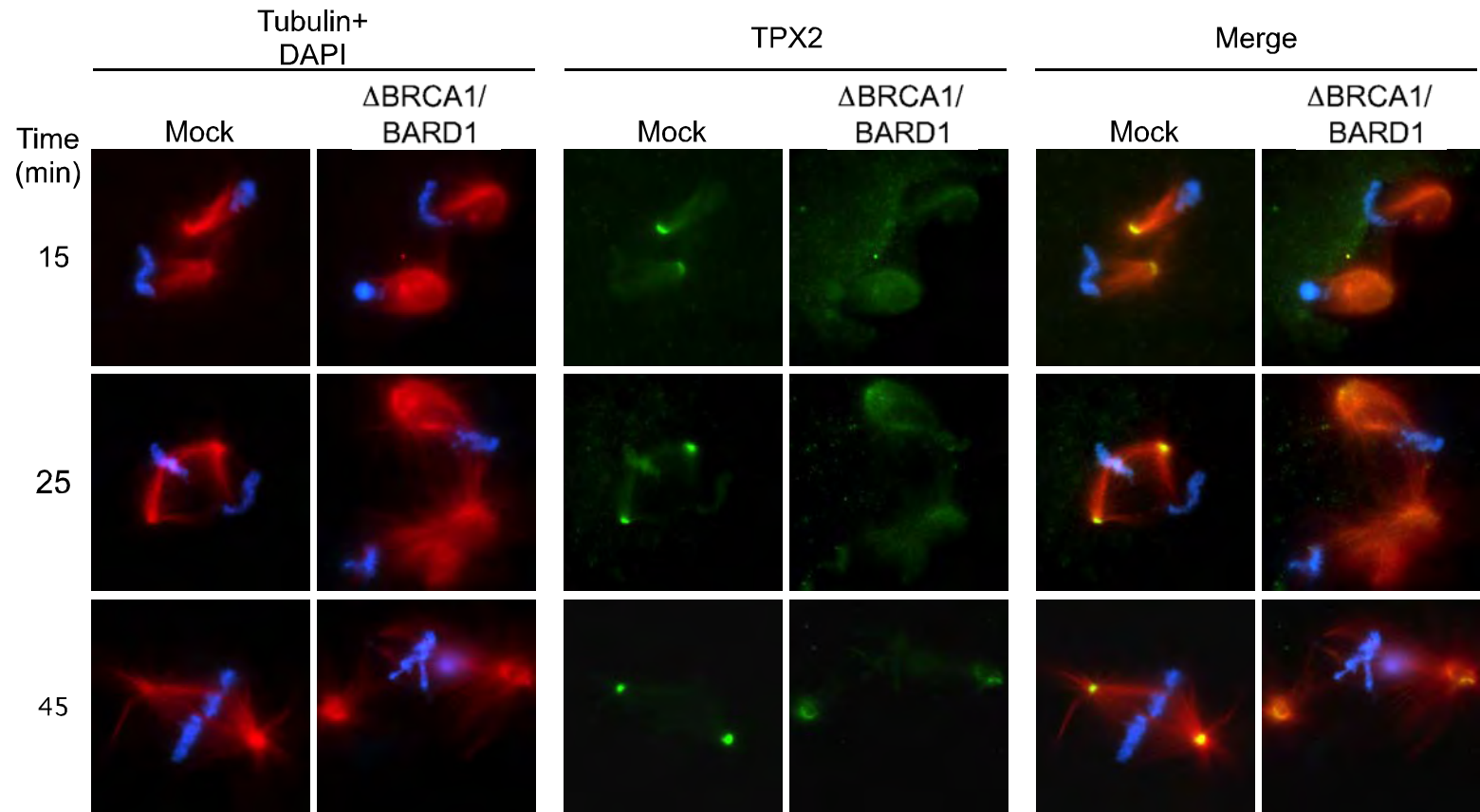


Figure 8. Chromatin-induced MT asters and spindles assembled in the mock-treated and the BRCA1/BARD1-depleted extracts (time course)

Metaphase-arrested extracts were supplemented with rhodamine-labeled tubulin, Alexa-Fluor-488-labeled TPX2 Ab, and sp chromatin. Aliquots of extracts were withdrawn at the indicated times, fixed, and analysed by IF microscopy. Representative mitotic figures are shown for each time point. Note unfocused aster/spindle poles and failure of TPX2 to accumulate on cent of these poles in BRCA1/BARD1-depleted extract.

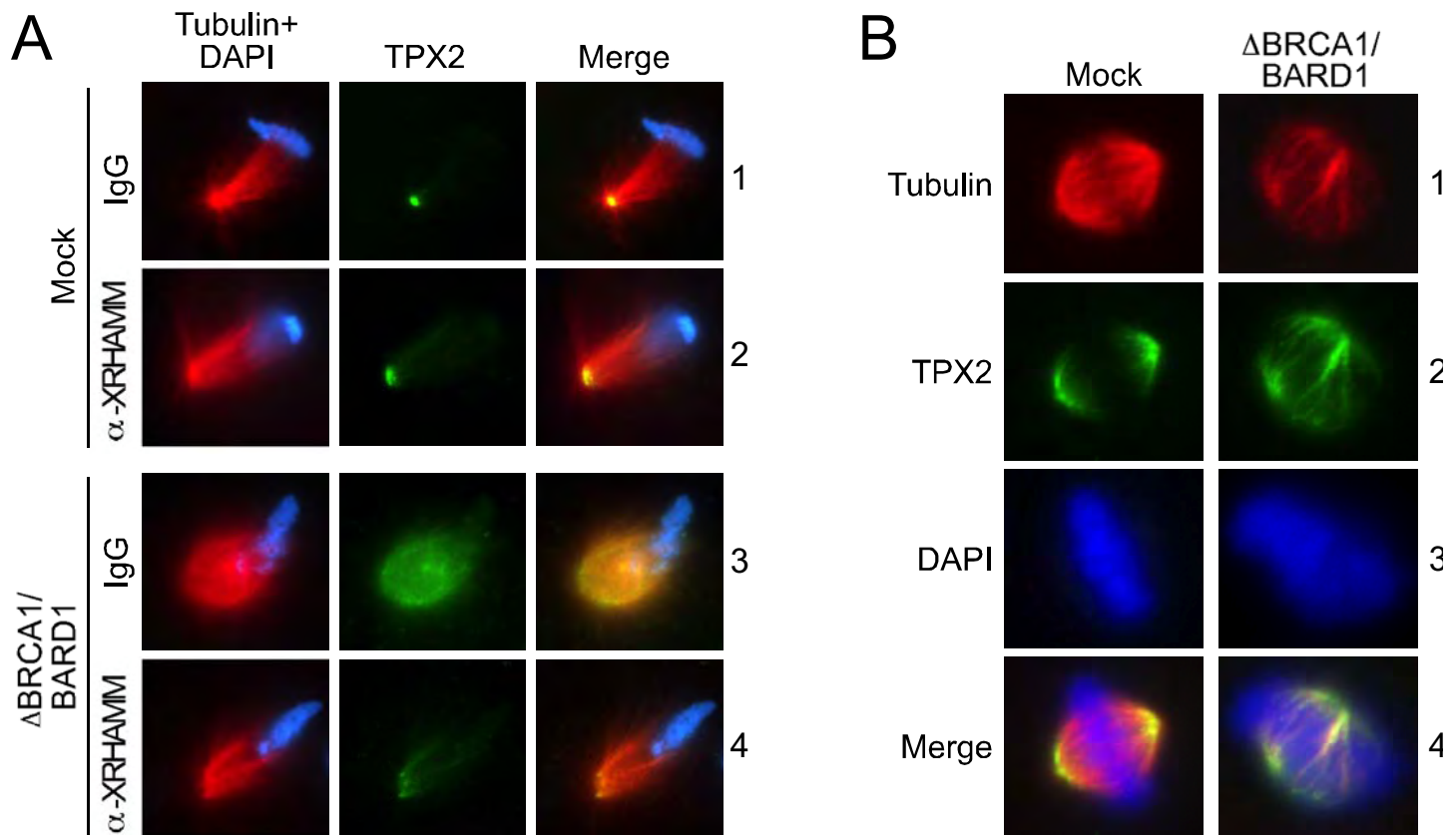


Figure 9. BRCA/BARD1 controls accumulation of TPX2 on spindle poles. (A) Representative chromatin-induced asters assembled in mock-treated and BRCA1/BARD1-depleted extracts supplemented with rhodamine-labeled tubulin, Alexa-Fluor-488-labeled anti-TPX2 Ab, and the indicated components. Notice rescue of spindle pole organization and the accumulation of TPX2 on spindle poles with the anti-XRHAMM antibody. (B) Representative IF images of TPX2 localization on metaphase spindles in HeLa cells transfected with control or BRCA1- and BARD1-specific siRNA.

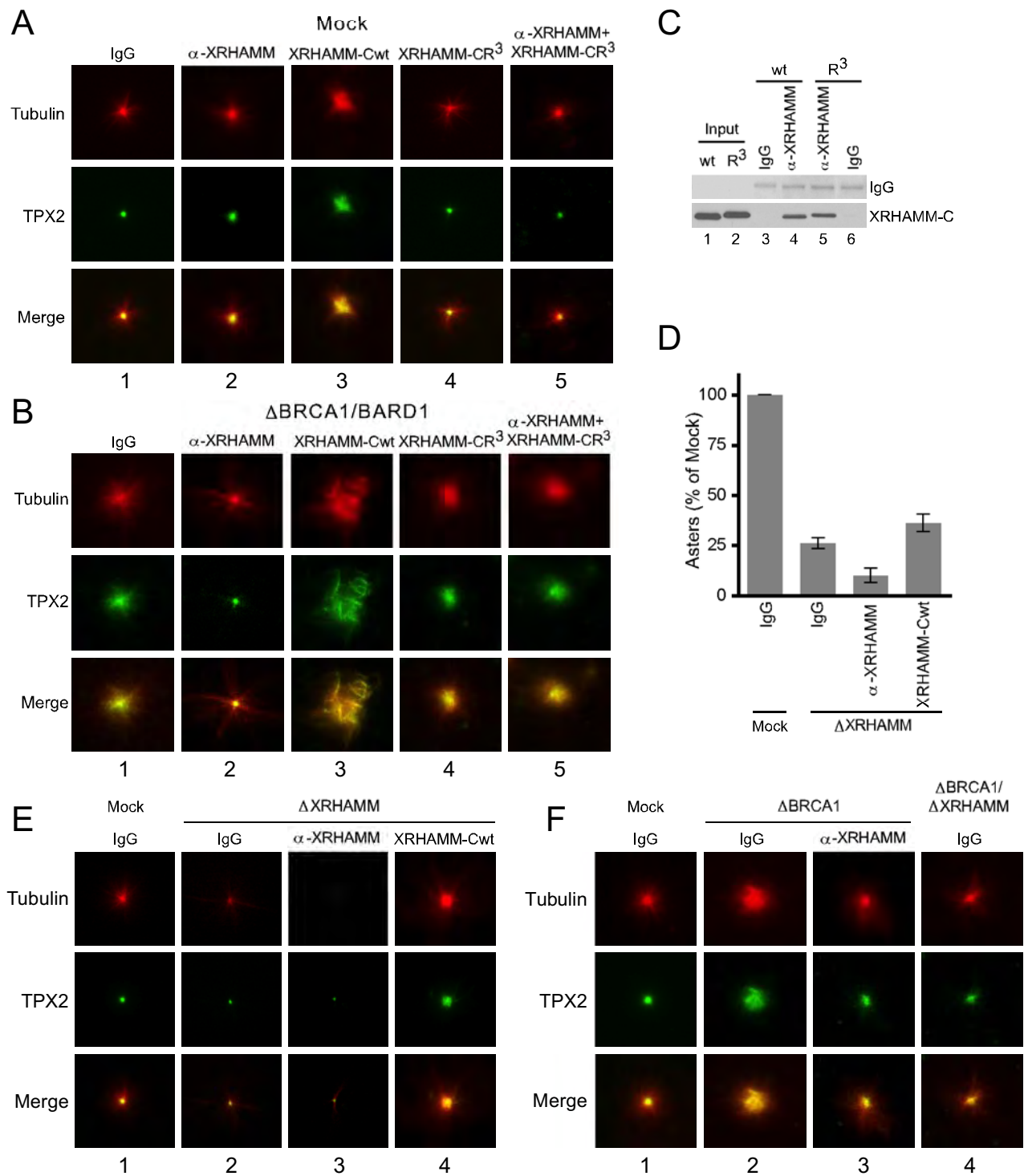


Figure 10. BRCA1/BARD1 regulates MT assembly in a XRHAMM-dependent fashion

Prior to assaying egg extracts, they were supplemented with rhodamine-labeled tubulin and Alexa-Fluor-488-labeled anti-TPX2 Ab; MT asters were induced by the addition of Ran(Q69L)-GTP (Except 'C'). (A and B) Representative asters assembled in mock-treated (A) and BRCA1/BARD1-depleted (B) extracts supplemented with the indicated components.

(C) Disruption of its leucine zipper does not affect the XRHAMM-C interaction with the corresponding specific Ab. Equal amounts of recombinant XRHAMM-Cwt (wt) and XRHAMM-CR3 (R3) were immunoprecipitated with the affinity-purified \pm -XRHAMM (lanes 4 and 5 respectively) or non-immune IgG (lanes 3 and 6 respectively) and analyzed by SDS-PAGE followed by staining the membrane with

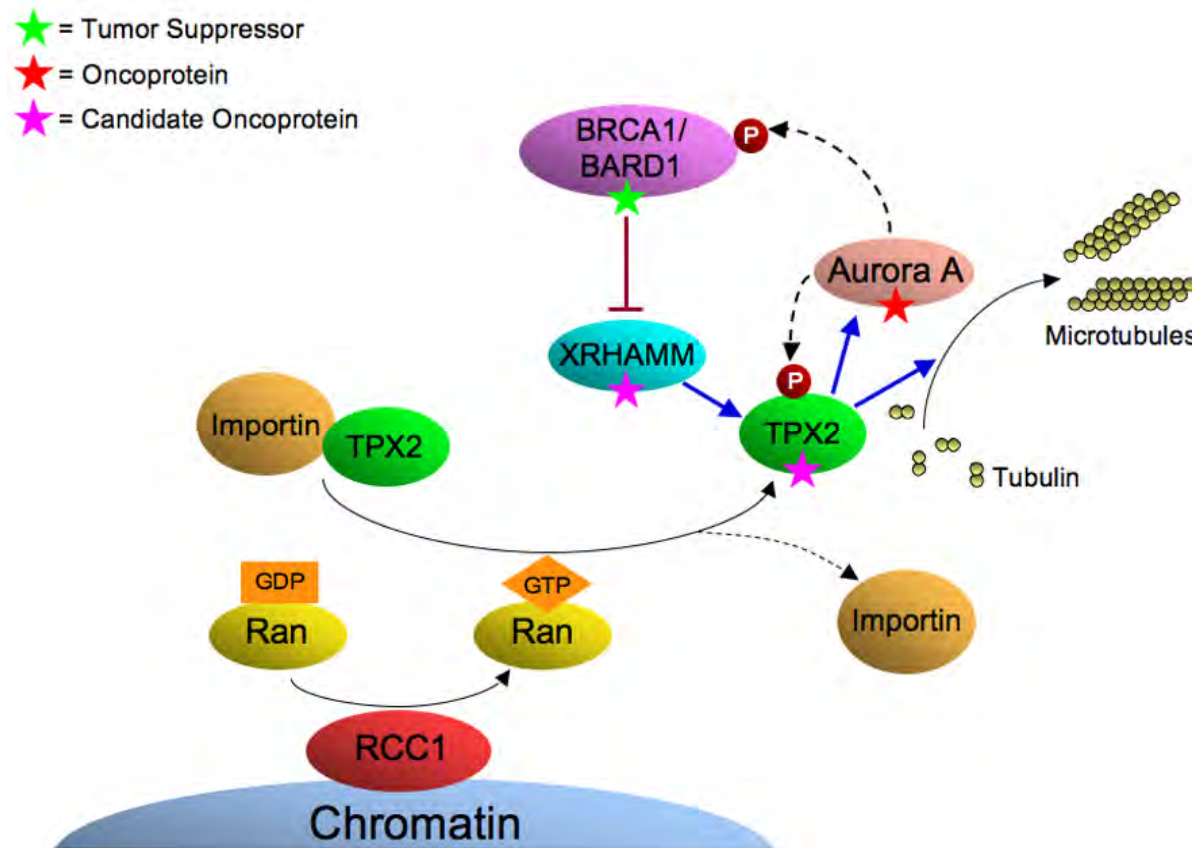


Figure 11. Schematic representation of BRCA1/BARD1 function during mitotic spindle assembly